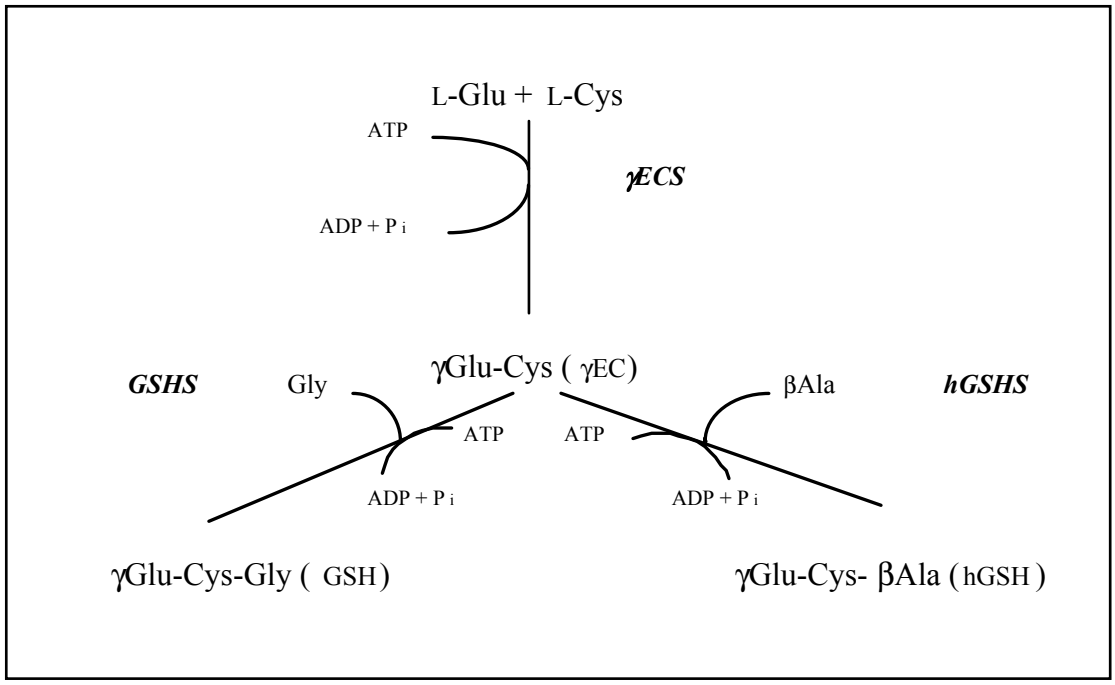
Tesis Doctoral

Síntesis de Glutatión y Homoglutatión en Nódulos de Leguminosas



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Tesis Doctoral

Síntesis de Glutatión y Homoglutatión en Nódulos de Leguminosas

Memoria presentada por D. Manuel Ángel Matamoros Galindo,

Licenciado en Ciencias, Sección Biológicas, para optar al grado de Doctor en Ciencias

Zaragoza, Junio de 2000

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CERTIFICA:

Que la Tesis Doctoral titulada "*Síntesis de Glutatión y Homoglutatión en Nódulos de Leguminosas*" ha sido realizada por el Licenciado en Ciencias Biológicas D. Manuel Ángel Matamoros Galindo en el Departamento de Nutrición Vegetal de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su dirección y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor en Ciencias.

Zaragoza, Junio de 2000

Dr. Manuel Becana Ausejo

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Dr. Carlos Gómez-Moreno Calera

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Abreviaturas

BSA	Seroalbúmina bovina
Cyt	Citocromo
dNTP	Desoxinucleótido trifosfato
DTE	Ditioeritritol
γEC	γ-glutamilcisteína
γECS	γ-glutamilcisteína sintetasa
GSH	Glutatión
GSHS	Glutatión sintetasa
GSSG	Glutatión oxidado
hGSH	Homoglutatión
hGSHS	Homoglutatión sintetasa
HPLC	Cromatografía líquida de alta resolución
Lb	Leghemoglobina
MBB	Monobromobimano
ORF	Marco de lectura
PEP	Fosfoenolpiruvato
pI	Punto isoeléctrico
plásmido Ti	Plásmido inductor de tumores
plasmido *vir*	Plásmido virulento
región T	Región transferida
SE	Error estándar
SOD	Superóxido dismutasa
T-DNA	DNA transferido
UTR	Región no traducida

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1. *Introducción general*

1.1. Fijación biológica del nitrógeno

El nitrógeno es uno de los elementos más ampliamente distribuidos en la Naturaleza. Es además de vital importancia para las plantas, donde se encuentra como el cuarto elemento más abundante, formando parte de numerosas biomoléculas como proteínas, ácidos nucleicos, porfirinas y alcaloides.

En la atmósfera se halla en forma de dinitrógeno (N_2), representando alrededor del 78% en volumen. En la litosfera la cantidad de nitrógeno es solamente un 25% de la presente en la atmósfera; además, el nitrógeno de la litosfera se encuentra en forma muy estable en las rocas, calculándose que sólo un 0,03% se encuentra en el suelo, y que de éste sólo una pequeña proporción está en forma asimilable por los seres vivos. El nitrógeno puede ser obtenido por las plantas por absorción del suelo en forma de NO_3^- y NH_4^+, o bien por reducción del N_2 atmosférico mediante asociación simbiótica con diversas bacterias (Tabla 1).

Tabla 1. *Grupos representativos y ejemplos de organismos fijadores de N_2 en simbiosis con plantas*

Simbiosis con leguminosas	*Rhizobium* *Sinorhizobium* *Bradyrhizobium* *Mesorhizobium* *Azorhizobium*	Guisante, judía, haba Alfalfa, *Medicago truncatula* Soja, lupino, "cowpea" *Lotus japonicus* *Sesbania*
Simbiosis actinorrícicas	*Frankia*	*Alnus, Casuarina, Myrica...*
Otras simbiosis	*Nostoc* *Anabaena*	Asociaciones de cianobacterias con angiospermas, gimnospermas, pteridófitos, briófitos y hongos.
Simbiosis asociativas	*Azotobacter* *Azospirillum notatum,*	Simbiosis asociativas y asociaciones casuales con raíces de *Paspalum* maíz...

La principal vía de producción de fertilizantes nitrogenados es la reacción Haber-Bosch, mediante la cual el N_2 es reducido a NH_4^+ por el H_2 a temperaturas y presiones muy elevadas. La utilización de fertilizantes conlleva un gasto considerable, al ser su producción dependiente de la energía liberada de los

combustibles fósiles, y constituye un riesgo potencial de contaminación y eutrofización de las aguas dulces por lixiviación del NO_3^- de los suelos. La fijación biológica de N_2 representa una alternativa económica y ecológicamente limpia frente a la fijación química. No obstante, los beneficios potenciales de la fijación biológica de N_2 en la agricultura no podrán ser aprovechados en su totalidad hasta que no se conozcan en profundidad los factores bióticos y abióticos que influyen sobre ella, así como sus mecanismos de actuación.

1.2. Simbiosis rizobio-leguminosa

Las simbiosis fijadoras de N_2 más conocidas e importantes desde un punto de vista agronómico son las que se establecen entre las raíces de las leguminosas y bacterias de los géneros *Rhizobium, Sinorhizobium* y *Bradyrhizobium* (designadas colectivamente como rizobios) (Fig.1).

Inicialmente, los rizobios se multiplican en la rizosfera, donde su crecimiento es generalmente favorecido frente al de otros microorganismos por sustancias nutritivas y factores de crecimiento presentes en los exudados de la raíz. Por ejemplo, la secreción de homoserina por las raíces de guisante favorece el crecimiento de *Rhizobium leguminosarum*, ya que este aminoácido es una excelente fuente de C y N para esta especie (Egeraat, 1975). Posteriormente, los rizobios son atraídos hacia la superficie radical mediante quimiotaxis, producida por flavonoides en concentraciones del orden nanomolar. Puede haber cierta especificidad entre el tipo de flavonoide liberado por la planta y la respuesta de una determinada especie de rizobio (Phillips y cols., 1990). Estos mismos flavonoides, en concentración micromolar, activan los genes responsables de la nodulación o genes *nod*.

Tras el reconocimiento específico entre polisacáridos de la pared celular de la bacteria y glicoproteínas producidas por la planta (lectinas), se produce la adhesión de las bacterias a la raíz. Un paso previo a este reconocimiento parece ser una unión no específica mediada por una proteína de 14 kDa, ampliamente distribuida no sólo en los rizobios, sino también en *Agrobacterium* (Kijne y cols., 1990). La proteína, denominada rhicadhesina, es capaz de unirse al Ca^{2+} *in vitro* y parece facilitar la adhesión del rizobio a las raíces (Smit y cols., 1987). Una vez que los rizobios se han unido a los pelos radicales, las bacterias penetran a través de la pared celular, quedando envueltas en una estructura tubular, conocida como cordón de infección, que progresa hacia la base del pelo radical. La presencia de un factor difusible del rizobio provoca el comienzo de las divisiones corticales, tanto en el córtex externo como en el córtex interno de la raíz. La localización de estas divisiones iniciales

determina que el nódulo sea de tipo indeterminado (con meristemo persistente) o determinado (sin meristemo persistente).

Figura 1. *Detalle de nódulos de cowpea (tipo determinado).*

1.2.1. Nódulos determinados e indeterminados

Dependiendo de la planta huésped el desarrollo nodular puede seguir dos patrones básicos (Tabla 2).

Tabla 2. *Características de los nódulos con crecimiento determinado e indeterminado*

	Determinado	Indeterminado
Planta huésped	Soja, judía, "cowpea"	Alfalfa, guisante, haba
Origen geográfico	Tropical y subtropical	Templado
Forma del nódulo	Esférica	Cilíndrica, a menudo ramificada
Lugar de inicio de las divisiones celulares	Córtex externo	Córtex interno
Crecimiento nodular	Expansión celular	División celular
Inductores de genes *nod*	Isoflavonas	Flavonas, flavononas
Producto exportado	Ureidos	Amidas

Datos compilados de Sprent (1980) y Hirsch (1992).

Nódulos indeterminados

Los nódulos de tipo indeterminado (Fig. 2) se caracterizan por la presencia de un meristemo nodular persistente (zona I), constituido por un grupo de células que se dividen activamente por mitosis. Algunas de las células derivadas permanecerán como parte del meristemo, mientras que otras se diferenciarán en tipos celulares específicos. La existencia de un meristemo persistente provoca que los nódulos de tipo indeterminado tengan generalmente forma alargada, ya que se forman continuamente nuevas células en el extremo distal del nódulo. Esto hace que todos los estadíos del desarrollo nodular estén representados en un único nódulo, existiendo un gradiente de edad desde la zona apical o distal (células más jóvenes) a la zona basal o proximal (células más viejas).

Cerca del meristemo nodular se encuentra la zona de invasión. Algunas células de esta zona son invadidas por cordones de infección, y en general son células más grandes y con mayor número de vacuolas que las células meristemáticas.

En el borde proximal de la zona de invasión se encuentran los bacteroides recientemente liberados de los cordones de infección. Éstos tienen forma alargada, todavía conservan la capacidad de dividirse y están rodeados por la membrana peribacteroidal, correspondiendo a los bacteroides de tipo 1.

A continuación se encuentra una zona de células más grandes, vacuoladas y diferenciadas que las de la zona anterior, denominada zona de simbiosis temprana, asignándose el término de zona prefijadora o zona II al conjunto formado por la zona de invasión y la zona de simbiosis temprana. Aquí se encuentran los bacteroides de tipo 2, más alargados que los de tipo 1.

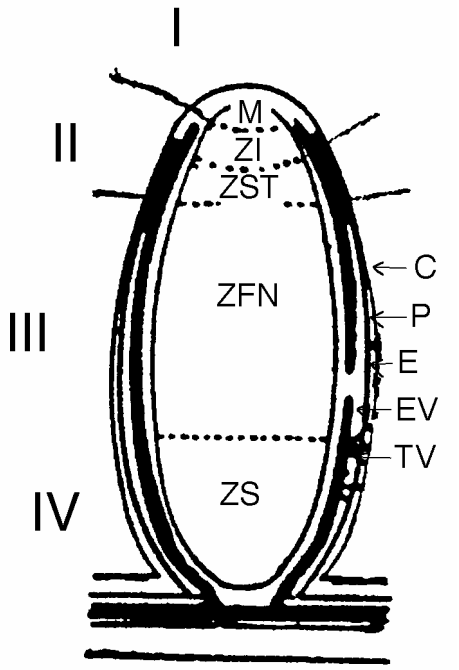


Figura 2. *Estructura general de un nódulo indeterminado (según Vasse y cols., 1990; Hirsch, 1992). M, meristemo; ZI, zona de invasión; ZST, zona de simbiosis temprana; ZFN, zona de fijación de nitrógeno; ZS, zona senescente; C, córtex nodular; P, parénquima nodular; E, endodermis; EV, endodermis vascular; TV, tejido vascular.*

En el borde proximal de la zona prefijadora se sitúa la interzona II-III, una estrecha franja de tejido de células grandes, que contienen gran cantidad de amiloplastos y bacteroides de tipo 3. En nódulos de alfalfa se ha detectado mRNA de leghemoglobina (Lb) en esta zona (Hirsch, 1992). La interzona II-III es una zona de transición en la expresión de genes necesarios para el inicio de la fijación de N_2.

En la zona de fijación de nitrógeno o zona III existen células no infectadas y células infectadas (repletas de bacteroides). La zona III puede a su vez subdividirse en una zona fijadora y una zona ineficiente, donde la tasa de fijación de N_2 se halla

sensiblemente reducida. En la zona fijadora se encuentran los bacteroides de tipo 4, caracterizados por una marcada heterogeneidad citoplasmática. Los bacteroides de tipo 5, presentes en la zona ineficiente, poseen en cambio un citoplasma más homogéneo. Finalmente, la zona senescente o zona IV, en la región proximal del nódulo, contiene las células más viejas, y se caracteriza por la presencia de pigmentos de degradación de la Lb. Estos pigmentos tienen una estructura química similar a la biliverdina animal y confieren una tonalidad verdosa a esta región del nódulo.

Rodeando a la zona de invasión y al resto de la región central del nódulo podemos encontrar otros tipos celulares formando parte de los tejidos vasculares, endodermis y parénquima nodular.

Nódulos determinados

Al igual que los nódulos indeterminados los nódulos determinados también pueden dividirse en diferentes regiones. Distinguimos así una región central o zona infectada, donde se produce la fijación de N_2, de una región externa, que incluye córtex, endodermis y parénquima nodular (Fig. 3).

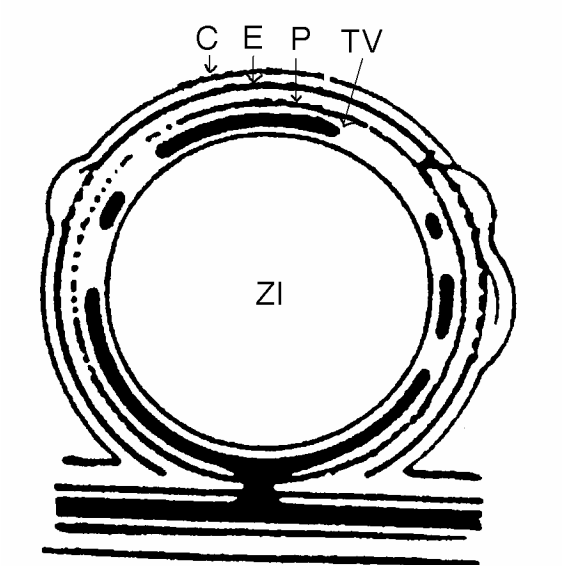


Figura 3. *Estructura general de un nódulo determinado (según Hirsch, 1992). ZI, zona infectada; C, córtex nodular; P, parénquima nodular; E, endodermis; TV, tejido vascular.*

El córtex constituye la zona exterior del nódulo y proviene de las células corticales de la raíz que inicialmente rodeaban el primordio nodular. En el parénquima se encuentran los haces vasculares y ambas zonas, córtex y parénquima, están separadas por la endodermis.

En los nódulos determinados existen dos regiones meristemáticas diferenciadas, y la mayoría de las divisiones en la región central del nódulo cesan entre 12 y 18 días después de la inoculación. El posterior aumento del tamaño nodular se da por crecimiento de las células ya existentes. Algunas células de esta región central son invadidas por cordones de infección. Otras, sin embargo, permanecen sin infectar, contituyendo las llamadas células intersticiales. Estas células son de menor tamaño que las infectadas y contienen gran cantidad de vacuolas. Las células intersticiales contienen en los peroxisomas una uricasa específica de nódulos (nodulina-35) (van den Bosch y Newcomb, 1986), y superan en número a las células infectadas en una proporción aproximada de 3 a 2. La función principal de las células intersticiales de los nódulos determinados es la síntesis de los ureidos alantoína y ácido alantoico (Sprent, 1980).

1.2.2. Nitrogenasa

La fijación biológica del N_2 es catalizada por el complejo enzimático nitrogenasa, presente exclusivamente en procariotas, según la reacción:

$$N_2 + 16\ ATP + 10\ H^+ + 8\ e^- \longrightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$$

En condiciones fisiológicas los electrones son utilizados para reducir el N_2 a NH_4^+ y, en menor cuantía, H^+ a H_2. La reacción catalizada por la nitrogenasa requiere un donador de electrones, ATP, Mg^{2+} y una concentración extremadamente baja de O_2. El donador de electrones de la nitrogenasa *in vivo* es una proteína de potencial redox muy negativo, tipo flavodoxina (en fijadores de vida libre como *Klebsiella*) o ferredoxina (en los fijadores simbióticos). No se conoce con seguridad cómo son reducidas, a su vez, la flavodoxina o la ferredoxina, pero probablemente el poder reductor necesario para ello está acumulado en forma de potencial de membrana o de gradiente de H^+.

La nitrogenasa está regulada *in vivo* a diversos niveles: transcripción, traducción, disponibilidad de sustrato, modificación covalente y moduladores alostéricos. El ADP-Mg^{2+} y el H_2, productos de la actividad nitrogenasa, son también potentes inhibidores del enzima purificado. Asimismo, el O_2 inactiva irreversiblemente el enzima.

1.3. Producción de especies reactivas de oxígeno

Todos los organismos aerobios deben hacer frente a un problema común. El consumo respiratorio de O_2 puede dar lugar a procesos oxidativos, debidos a la formación de peróxido de hidrógeno (H_2O_2) y radicales libres superóxido (O_2^-) e hidroxilo (·OH).

En las plantas la situación es particularmente crítica, ya que además de utilizar el O_2 como aceptor final de electrones en la respiración, éste es producido durante la fotosíntesis. En los cloroplastos se puede generar también oxígeno singlete ($^1O_2$), altamente reactivo, por transferencia de la energía de excitación desde la clorofila en estado triplete al oxígeno.

Los radicales libres (O_2^-,·OH), peróxidos (H_2O_2, peróxidos orgánicos) y el $^1O_2$ se incluyen dentro de las denominadas especies de oxígeno activado o, como se utilizará en esta Tesis, especies reactivas de oxígeno.

1.3.1. En la fotosíntesis

Durante la fase luminosa de la fotosíntesis la energía de la luz es utilizada para romper una molécula de agua según la reacción de Hill:

$$H_2O \longrightarrow 1/2\ O_2 + 2\ H^+ + 2\ e^-$$

Los electrones liberados, tras ser elevados a niveles más energéticos por los procesos fotoquímicos del fotosistema II, pasan a través de una serie de transportadores (el conocido esquema en Z), reduciendo finalmente el $NADP^+$ a NADPH en una reacción catalizada por la ferredoxina-$NADP^+$ reductasa. El potencial redox de la ferredoxina (-0,43 V) es casi idéntico al requerido para reducir el O_2 a O_2^-, por lo que no es sorprendente que parte de los electrones de la ferredoxina sean desviados a la formación de O_2^- (Misra y Fridovich, 1971). El radical O_2^- puede también producirse sin la intervención de la ferredoxina, por reducción directa del O_2 con electrones provenientes del fotosistema I (Elstner, 1982). Otra especie altamente reactiva de oxígeno es el $^1O_2$, que, como se mencionó anteriormente, puede generarse en el interior de los cloroplastos cuando una molécula de clorofila transfiere directamente al O_2 la energía de excitación.

1.3.2. En la respiración

Las especies reactivas de oxígeno se generan también en las mitocondrias de todos los organismos aerobios. En las plantas la situación es especialmente complicada, debido a la frecuente presencia de ramificaciones en la cadena de transporte electrónico y a la aparición de más de una oxidasa terminal.

En el último paso de la cadena de transporte electrónico el O_2 es reducido a H_2O con la transferencia de cuatro electrones:

$$O_2 + 4\ e^- + 4\ H^+ \longrightarrow 2\ H_2O$$

En general, esta reacción es catalizada por la citocromo oxidasa, un enzima muy eficiente que reduce O_2 a H_2O sin generar intermediarios potencialmente tóxicos como el radical O_2^- o el H_2O_2. Sin embargo, la transferencia de electrones de un transportador al siguiente en la cadena no es siempre tan eficiente. Por ejemplo, la flavodoxina-NADH deshidrogenasa puede reducir directamente el O_2 a O_2^- (Rich y Bonner, 1978).

1.3.3. En la fijación de N_2

La fijación de N_2 en los nódulos de leguminosas requiere mecanismos para hacer frente a la toxicidad de las especies reactivas de oxígeno.

La nitrogenasa es extremadamente sensible al O_2. Las razones últimas de esta sensibilidad no están claras, pero es probable que el enzima sea capaz de reducir el O_2 a O_2^- o a H_2O_2, que destruirían el enzima. La evidencia de este mecanismo es indirecta. Así, la actividad nitrogenasa es parcialmente protegida *in vitro* de la inactivación en presencia de O_2 por enzimas que eliminan el O_2^- y el H_2O_2 (Mortensen y cols., 1974; Robson y Postgate, 1980).

Para evitar la inhibición de la fijación de N_2 por el O_2, los nódulos de leguminosas contienen gran cantidad de Lb, una proteína monomérica de 16 kDa que transporta O_2 y que tiene una estructura similar a la mioglobina de mamíferos (Appleby, 1984). La Lb mantiene en la zona infectada del nódulo una concentración de O_2 de 10-50 nM, lo cual evita la inhibición de la nitrogenasa a la vez que asegura el aporte del O_2 necesario para la respiración de los bacteroides. Sin embargo, la Lb constituye una de las principales fuentes de especies reactivas de oxígeno en el interior de los nódulos. Así, la Lb oxigenada es susceptible de autoxidación. En este

proceso, favorecido por el pH ligeramente ácido de los nódulos (Becana y Klucas, 1990), se genera O_2^- y H_2O_2 (Puppo y cols., 1981). Ambas especies son altamente tóxicas para las células, pero su mayor peligrosidad reside en la producción del radical ·OH, extremadamente oxidante, mediante la reacción Haber-Weiss catalizada por Fe (Halliwell y Gutteridge, 1999):

$$H_2O_2 + Fe^{2+} \longrightarrow {\cdot}OH + OH^- + Fe^{3+}$$
$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + O_2^- \longrightarrow {\cdot}OH + OH^- + O_2$$

El H_2O_2 ataca el grupo hemo de la Lb, liberándose átomos de Fe que catalizan la formación del radical ·OH (Puppo y Halliwell, 1988). El H_2O_2 puede además reaccionar tanto con la forma ferrosa (Fe^{2+}) como con la forma férrica (Fe^{3+}) de la Lb, oxidándolas a ferril-Lb (Aviram y cols., 1978; Puppo y cols., 1993). Las formas oxidadas (Lb^{3+} y ferril-Lb) son incapaces de transportar O_2.

En los nódulos de leguminosas existen al menos cuatro mecanismos para convertir la Lb de nuevo a la forma fisiológicamente activa :

1. La Lb^{3+} reductasa cataliza la reducción de Lb^{3+} a Lb^{2+} utilizando NADH como poder reductor (Ji y cols., 1991).

2. A concentraciones fisiológicas el ascorbato podría contribuir a la reducción directa de la Lb^{3+} (Becana y Klucas, 1990).

3. La reducción de la Lb^{3+} mediada por flavinas (principalmente riboflavina, aunque también FMN y FAD), podría ser importante a las pequeñas concentraciones de O_2 existentes en los nódulos. El NAD(P)H reduce las flavinas, que a su vez reducirían el Fe^{3+} del grupo hemo (Becana y cols., 1991).

4. Los nódulos de soja, judía y cowpea contienen pequeñas moléculas de naturaleza desconocida, que reducen eficientemente la Lb^{3+} en presencia de NAD(P)H por medio de una reacción en la que interviene el radical O_2^- (Becana y Klucas, 1990).

En los nódulos existen, además de la Lb, otras fuentes potenciales de especies reactivas de oxígeno:

Respiración: los nódulos de leguminosas poseen altas tasas respiratorias, debido a la gran demanda de energía originada por la fijación de N_2. Como en otros tejidos, las mitocondrias de los nódulos generan O_2^- y H_2O_2 como consecuencia inevitable de la respiración.

Ferredoxina: la ferredoxina, el reductor fisiológico de la nitrogenasa, puede generar el radical O_2^- de forma similar a como se describió en cloroplastos.

Hidrogenasa: la mayoría de los rizobios contienen hidrogenasa, un enzima asociado a membrana que cataliza la oxidación de H_2 a H_2O. Su actividad catalítica puede dar lugar también a la producción de O_2^- (Schneider y Schlegel, 1981).

Uricasa: los nódulos determinados contienen en general grandes cantidades de uricasa. Este enzima está localizado en los peroxisomas, donde participa en la biosíntesis de ureidos, pudiendo dar lugar a la formación de cantidades importantes de H_2O_2 (van den Bosch y Newcomb, 1986).

1.3.4. En condiciones de estrés

Las plantas se encuentran expuestas a numerosos estreses ambientales, tanto naturales (sequía, temperaturas extremas, ataque por microorganismos e insectos), como relacionados con la actividad humana (contaminación ambiental). Estos estreses, aislados o combinados, tienen consecuencias negativas y son el principal factor limitante de la producción vegetal (Allen, 1995). Aunque el mecanismo de acción de muchos de ellos es todavía desconocido, gran parte de las alteraciones metabólicas y fisiológicas causadas por la exposición a estos estreses están asociadas con el daño oxidativo a nivel celular y molecular, y son consecuencia de la producción incontrolada de especies reactivas de oxígeno (Thompson y cols., 1987a; Smirnoff, 1993; Moran y cols., 1994).

1.4. Estrés oxidativo

Para que se mantenga la eficiencia metabólica y funcional de la célula es necesario que exista un equilibrio entre la producción y la destrucción de especies reactivas de oxígeno (Foyer y cols., 1994; Halliwell y Gutteridge, 1999). Sin embargo, en determinadas situaciones como la senescencia natural o inducida por estrés, el balance entre prooxidantes y defensas antioxidantes se desequilibra en favor de los primeros, dando lugar a un estrés oxidativo. Éste se manifiesta por la oxidación de las biomoléculas fundamentales (azúcares, lípidos, proteínas, DNA y vitaminas) y está causado por diversas especies reactivas de oxígeno, especialmente por el radical ·OH (Halliwell y Gutteridge, 1999).

1.4.1. Daño oxidativo a proteínas

Las proteínas participan en una gran variedad de procesos *in vivo* que incluyen el mantenimiento de la estructura de las membranas y del citoesqueleto, la catálisis de las reacciones metabólicas, el transporte de metabolitos, la interacción con receptores, y el control del crecimiento y desarrollo de la planta.

La modificación oxidativa y posterior degradación de proteínas como consecuencia del ataque por radicales libres altera el funcionamiento normal de la célula (Levine y cols., 1990). La oxidación de proteínas origina un aumento en los grupos carbonilo (Moran y cols., 1994) y la aparición de aminoácidos modificados, como la 2-oxohistidina, el metionil-sulfóxido y la cistina (Murphy y Kehrer, 1989; Stadtman, 1992; Ferguson y Burke, 1994). Las proteínas expuestas al radical ·OH muestran alteraciones en su estructura y pueden sufrir fragmentaciones espontáneas o ser más susceptibles a la proteolisis (Davies, 1987). Los residuos de His, Tyr, Phe, Trp, Met y Cys son los más frecuentemente atacados por los radicales libres, siendo la Pro, Arg y Lys los más sensibles a la oxidación a grupos carbonilo (Stadtman, 1992).

La formación de grupos carbonilo mediante modificación oxidativa ha sido profusamente estudiada en animales y humanos y está asociada con el envejecimiento y diversos estados patológicos. En las plantas se ha observado un aumento del nivel de proteínas oxidadas en hojas (Moran y cols., 1994; Iturbe-

Ormaetxe y cols., 1998) y nódulos (Escuredo y cols., 1996; Gogorcena y cols., 1995, 1997) de leguminosas sometidas a diferentes estreses ambientales.

1.4.2. Daño oxidativo a lípidos

En las células vegetales existe una gran variedad de lípidos. Los más comunes son los ácidos grasos, fosfolípidos, ceras y terpenos. Los ácidos grasos no sólo actúan como reserva metabólica, sino que además forman parte de los fosfolípidos de membrana, regulando la fluidez de ésta. Los ácidos grasos poliinsaturados son particularmente susceptibles al ataque por radicales, generando peróxidos de lípidos, como por ejemplo:

$$\begin{array}{c} \text{O-OH} \\ | \\ CH_3CH{=}CH\text{-}CH(CH_2)_nCOOH \end{array}$$

La peroxidación de los ácidos grasos insaturados conlleva alteraciones importantes en la estructura y función de las membranas. Estas alteraciones pueden provocar la descompartimentación de iones y la pérdida del potencial de membrana, la inhibición del transporte de metabolitos, modificaciones en los receptores de hormonas, y la producción de mensajeros químicos "de alarma" como consecuencia de la oxidación incontrolada de lípidos y proteínas de membrana. Todas estas modificaciones conducen, en último término, a la senescencia y muerte celular (Thompson y cols., 1987b).

La peroxidación de lípidos se inicia con la abstracción de un átomo de hidrógeno por un radical libre, a partir de un grupo metilo de un ácido graso poliinsaturado (Mead, 1976; Sevanian y Hochstein, 1985). El proceso es una reacción en cadena autopropagante, y los hidroperóxidos de lípidos resultantes pueden descomponerse fácilmente en especies reactivas como los radicales alcoxilo, alcanos, epóxidos de lípidos, alcoholes y aldehídos citotóxicos como el malondialdehído. Estos productos de la peroxidación de lípidos pueden formar aductos con las proteínas (Uchida y Stadtman, 1993; Uchida y cols., 1993) y el DNA (Chaudhary y cols., 1994), dando lugar a mutaciones y alteraciones de la expresión génica. De hecho, la peroxidación lipídica ha sido empleada como un marcador de estrés oxidativo tanto en tejidos animales como vegetales (Halliwell y Gutteridge, 1999).

1.4.3. Daño oxidativo a DNA

El DNA también puede sufrir modificaciones en condiciones de estrés oxidativo. Aunque ni el O_2^- ni el H_2O_2 reaccionan con el DNA, el radical ·OH sí puede hacerlo, provocando la rotura de las cadenas de DNA y la oxidación de la desoxirribosa y de las bases, lo que puede originar mutaciones (Fraga y cols., 1990; Halliwell y Aruoma, 1991; Wiseman y Halliwell, 1996; Evans y cols., 1999).

1.5. Mecanismos de protección frente al estrés oxidativo

Los bacteroides requieren O_2 para sintetizar el ATP necesario para los procesos biosintéticos y la fijación de N_2. Sin embargo, el O_2 inhibe la actividad de la nitrogenasa, por lo que los nódulos deben poseer mecanismos que mantengan el O_2 a una concentración suficientemente baja, pero constante, que permita simultáneamente tasas elevadas de respiración y fijación de N_2.

Asimismo, las células vegetales cuentan con una amplia variedad de defensas antioxidantes que en condiciones óptimas evitan la aparición del estrés oxidativo. Entre estas defensas se encuentran tanto enzimas como metabolitos de bajo peso molecular, que destruyen las especies reactivas de oxígeno o, en algunos casos, evitan su formación.

1.5.1. Barrera a la difusión de oxígeno

En los nódulos de leguminosas el suministro de O_2 a la zona central o infectada está controlado por una barrera variable a la difusión de O_2 localizada en el córtex interno (Witty y cols., 1986). Aunque la estructura y el mecanismo bioquímico de la barrera a la difusión de oxígeno no se conocen con exactitud, se acepta de modo general que esta barrera es esencial para regular la difusión de O_2 en respuesta a los cambios ambientales, para evitar el daño oxidativo en la zona central del nódulo y, en suma, para mantener una óptima fijación de N_2.

1.5.2. Leghemoglobina

Una vez que el O_2 atraviesa la barrera y alcanza la zona central de los nódulos, la Lb lo transporta desde la membrana plasmática de las células infectadas a la membrana del simbiosoma. Aparentemente, el O_2 libre difunde entonces a través del espacio peribacteroidal, que carece de Lb, para alcanzar finalmente las oxidasas terminales de alta afinidad de los bacteroides. La difusión facilitada de O_2 por la Lb y las altas tasas respiratorias de los bacteroides aseguran que el flujo de O_2 sea bajo y constante a través del citoplasma vegetal, evitando que se produzcan cambios bruscos en el suministro de O_2 que serían perjudiciales para la nitrogenasa (Appleby, 1984).

1.5.3. Defensas enzimáticas

Superóxido dismutasa

En las plantas la eliminación del O_2^- se lleva a cabo mediante una reacción de dismutación catalizada por la superóxido dismutasa (SOD; EC 1.15.1.1):

$$O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

Existen tres tipos de SODs dependiendo del metal que utilizan como cofactor: cobre y zinc (CuZnSOD), manganeso (MnSOD) o hierro (FeSOD) (Salin, 1988; Bowler y cols., 1994). Los tres tipos de enzima son codificados en el núcleo y sintetizados en el citoplasma, siendo dirigidos a las mitocondrias, cloroplastos o peroxisomas dependiendo de su secuencia N-terminal y/o C-terminal. En general, las CuZnSODs se localizan en los cloroplastos y el citosol, las MnSODs en las mitocondrias y los peroxisomas y las FeSODs en los cloroplastos.

Mediante la eliminación del O_2^-, las SODs disminuyen el riesgo de formación del radical ·OH a través de la reacción Haber-Weiss. El H_2O_2 generado como producto de la reacción es eliminado por la catalasa o por la ascorbato peroxidasa en el ciclo ascorbato-glutatión (GSH).

Catalasa

La catalasa (EC 1.11.1.6) es una hemoproteína tetramérica ampliamente distribuida en las plantas, que cataliza la eliminación del H_2O_2 según la reacción:

$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

Al contrario que en las reacciones catalizadas por peroxidasas, la catalasa no requiere el aporte de poder reductor. Pero esta aparente ventaja está compensada por la baja afinidad (K_m~ 1M) de la catalasa por el H_2O_2 (Halliwell, 1982a), lo que determina que sea un enzima poco eficiente en la eliminación de bajas concentraciones de H_2O_2.

En las plantas la mayoría del enzima está localizado en orgánulos como los peroxisomas, donde su concentración es tan elevada que puede llegar a cristalizar. Su función en estos orgánulos es eliminar el H_2O_2 que producen varios enzimas, como la glicolato oxidasa y la uricasa. En estas condiciones de elevada concentración de H_2O_2 la catalasa resulta bastante efectiva. Se ha detectado un isoenzima de la catalasa, CAT-3, en mitocondrias de maíz (Scandalios y cols., 1980). Sin embargo, la catalasa está ausente en el citosol y en los cloroplastos, donde la eliminación del H_2O_2 es llevada a cabo por la ascorbato peroxidasa y quizás también por otras peroxidasas (Dalton, 1995).

Guaiacol peroxidasas

Las plantas superiores contienen varias peroxidasas que catalizan la reacción general:

$$H_2O_2 + RH_2 \longrightarrow 2\,H_2O + R$$

donde el RH_2 es el donador de electrones. Puesto que se desconoce cuál es el donador de electrones *in vivo*, estas peroxidasas se denominan "no específicas" o "guaiacol peroxidasas".

Las guaiacol peroxidasas tienen generalmente una masa molecular de ~ 50 kDa, y ocho residuos de Cys formando cuatro puentes disulfuro que no son destruidos por los reactivos de tioles (Asada, 1996). Las numerosas guaiacol peroxidasas existentes en los tejidos vegetales pueden eliminar el H_2O_2, pero su función parece estar relacionada más bien con procesos como la síntesis de lignina y etileno, y el crecimiento y morfogénesis de la planta (Campa, 1991).

Ciclo ascorbato-GSH

El ciclo ascorbato-GSH es uno de los principales sistemas antioxidantes en cianobacterias, cloroplastos y nódulos (Asada y Takahashi, 1987; Foyer y cols., 1994; Dalton, 1995). La función de este ciclo es eliminar el H_2O_2, utilizando en último

término el poder reductor del NAD(P)H. En la reacción inicial catalizada por la ascorbato peroxidasa (EC 1.11.1.11), el H_2O_2 es reducido a agua por el ascorbato (Fig. 4). El producto inicial es el monodeshidroascorbato (o radical ascorbato), que puede oxidarse espontáneamente a deshidroascorbato. El ascorbato puede ser regenerado a partir del monodeshidroascorbato por la monodeshidroascorbato reductasa (EC 1.6.5.4), con oxidación concomitante de NADH.

El ascorbato puede también ser regenerado a partir del deshidroascorbato por la deshidroascorbato reductasa (EC 1.8.5.1), utilizando GSH como reductor; a su vez, el GSH consumido puede regenerarse a partir de la forma oxidada (GSSG) por la acción de la glutatión reductasa (EC 1.6.4.2). Por el contrario, en *Chlamydomonas* y vertebrados no existe ciclo ascorbato-GSH, sino que la reducción inicial del H_2O_2 a agua es llevada a cabo por la glutatión peroxidasa. Este enzima no está presente en invertebrados, aunque recientemente se ha detectado en plantas superiores (Smith y Shrift, 1979; Yokota y cols., 1988; Eshdat y cols., 1997).

Figura 4. *Esquema del ciclo ascorbato-GSH. DHA, deshidroascorbato; MDHA, monodeshidroascorbato; APX, ascorbato peroxidasa; DR, deshidroascorbato reductasa; GR, glutatión reductasa; MR, monodeshidroascorbato reductasa.*

Como en el caso de los cloroplastos, los nódulos muestran elevadas actividades de los cuatro enzimas del ciclo ascorbato-GSH. Durante el desarrollo de los nódulos, las actividades ascorbato peroxidasa y deshidroascorbato reductasa se incrementan de un modo notable, y están correlacionadas positivamente con la actividad fijadora de N_2 y con el contenido de Lb (Dalton y cols., 1986). Esta correlación sugiere que la actividad del ciclo ascorbato-GSH es esencial para el mantenimiento efectivo de la fijación de N_2. Otras evidencias de la importancia del

ciclo ascorbato-GSH en la fijación de N_2 se obtuvieron al comparar los nódulos de cultivares con diferentes actividades fijadoras. Los nódulos eficientes mostraron actividades enzimáticas superiores a los ineficientes para los cuatro enzimas del ciclo. El contenido total de tioles también fue tres o cuatro veces superior en los nódulos eficientes, y éstos además poseían más NAD^+, $NADP^+$ y NADPH (Dalton y cols., 1993).

Otra evidencia indirecta del papel protector del ciclo ascorbato-GSH en la fijación de N_2 es la obtenida en experimentos con atmósferas hiperbáricas de O_2. Dalton y cols. (1991) observaron en nódulos de soja un incremento de los contenidos de ascorbato y GSH y de las actividades de los enzimas del ciclo cuando las raíces se exponían a 50% O_2. Estas observaciones pueden interpretarse asumiendo que el aumento en la concentración de O_2 externo incrementa la producción de especies reactivas de oxígeno, lo que a su vez induce la expresión de los genes que codifican los enzimas del ciclo ascorbato-GSH.

1.5.4. Antioxidantes no enzimáticos

Entre los metabolitos de bajo peso molecular con función antioxidante cabe destacar, por su importancia y amplia distribución, el ascorbato o vitamina C y el tripéptido GSH (γGlu-Cys-Gly), el principal tiol no proteico en la mayoría de animales, plantas y procariotas (Meister y Anderson, 1983; Hausladen y Alscher, 1993; Rennenberg, 1997). Las hojas, raíces y nódulos de algunas leguminosas pueden contener, además del GSH o sustituyéndolo, el tripéptido homoglutatión (hGSH; γ-Glu-Cys-βAla) (Klapheck, 1988).

Ascorbato

El ascorbato es el compuesto reductor hidrosoluble más abundante en todas las plantas superiores, aunque no se ha encontrado en semillas (Dalton, 1995). Su importancia radica en sus propiedades como antioxidante, pudiendo reaccionar con las diferentes especies reactivas de oxígeno (Halliwell, 1982b; Bendich y cols., 1986) y participando en el ciclo ascorbato-GSH. El mecanismo de acción implica la capacidad del anión ascorbato (forma desprotonada predominante en codiciones fisiológicas) para donar un electrón a las especies reactivas de oxígeno con formación de radical monodeshidroascorbato. La oxidación posterior del monodeshidroascorbato por cesión de un segundo electrón da lugar al deshidroascorbato (Fig. 5).

Los cloroplastos contienen una concentración de ascorbato de 10-50 mM (Halliwell, 1982b; Foyer y cols., 1983; Foyer, 1993). Esta alta concentración de ascorbato proporciona a las células vegetales una defensa importante frente a las especies reactivas de oxígeno. En los nódulos su concentración es de 1-2 mM (Dalton y cols., 1986; Gogorcena y cols., 1997).

Figura 5. *Estructura del ascorbato y de sus productos de oxidación. MDHA, monodeshidroascorbato; DHA, deshidroascorbato.*

1.6. Glutatión y homoglutatión

La mayor parte de nuestro conocimiento actual de los procesos implicados en el metabolismo del GSH proviene de investigaciones realizadas en animales y humanos en los inicios de la década de los 80 (Meister, 1981; Meister y Anderson, 1983). Sin embargo, durante los últimos años se ha puesto de manifiesto la gran importancia del GSH en la fisiología de las plantas bajo condiciones de estrés y se ha relacionado la tolerancia a diversos estreses ambientales con elevados niveles de GSH (Smith y cols., 1990; Rennenberg y Brunold, 1994; May y cols., 1998a). Asimismo, el aumento de la biosíntesis de GSH y de la actividad glutatión reductasa parece ser una respuesta de las plantas a situaciones de estrés.

Las principales características del GSH son las siguientes:

1. El GSH es un compuesto abundante y ampliamente distribuido en la mayoría de los seres vivos (Meister y Anderson, 1983; Hausladen y Alscher, 1993; Rennenberg, 1997). En los nódulos de leguminosas existen elevadas concentraciones de tioles (Dalton y cols., 1991; Escuredo y cols., 1996; Gogorcena y cols., 1995, 1997).

2. El potencial redox (-0,34 V) del GSH le permite reducir eficientemente el deshidroascorbato, así como los puentes disulfuro que establecen algunas proteínas, lo cual le otorga un papel clave en el control del estado redox celular.

3. El GSH actúa como un eficaz antioxidante destruyendo especies reactivas de oxígeno potencialmente dañinas, tanto de forma directa como por medio del ciclo ascorbato-GSH (Hausladen y Alscher, 1993; Rennenberg, 1997; May y cols., 1998a). La función como antioxidante reside en el grupo sulfidrilo de la Cys, el cual tras oxidarse establece un puente disulfuro con el grupo tiol de una segunda molécula de GSH para formar GSSG (Fig. 6).

4. La gran estabilidad del GSH es debida a que el enlace peptídico entre el Glu y la Cys se establece entre el carbono γ de la cadena lateral del Glu y el grupo amino de la Cys; esto evita que el tripéptido sea degradado por aminopeptidasas.

5. Algunas plantas contienen tripéptidos homólogos al GSH, en los que la Gly es sustituida por otros aminoácidos. Los más importantes de estos tripéptidos son el hGSH (Klapheck, 1988), hidroximetilglutatión (γGlu-Cys-Ser) (Klapheck y cols., 1992) y γGlu-Cys-Glu (Meuwly y cols., 1993). Tanto la forma oxidada del hGSH como la del hidroximetilglutatión son reducidas por la glutatión reductasa de levaduras (Klapheck, 1988; Klapheck y cols., 1992), lo cual sugiere que ambos tripéptidos desempeñan funciones similares al GSH tanto a nivel bioquímico como fisiológico.

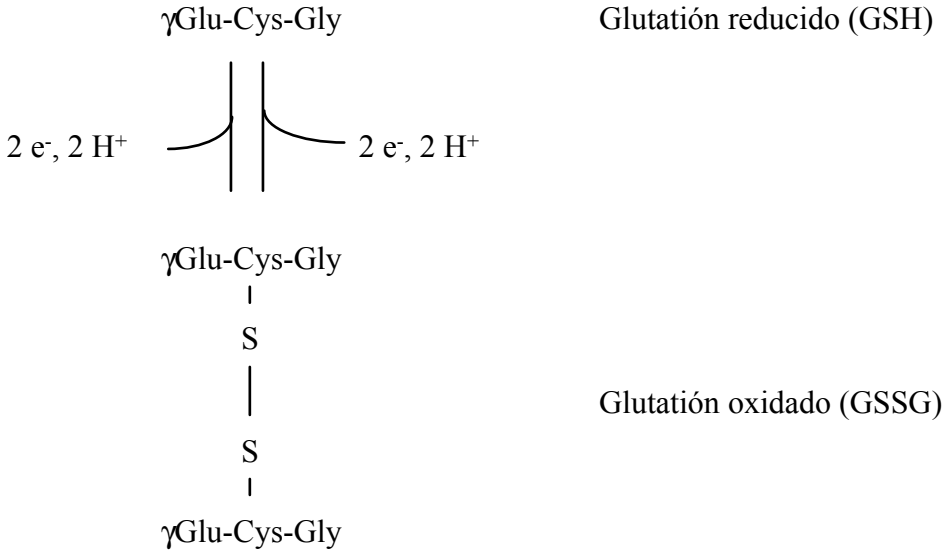


Figura 6. *Estructura de las formas reducida y oxidada del glutatión*

1.6.1. Biosíntesis

La síntesis de GSH tiene lugar en las células vegetales mediante dos reacciones secuenciales que son probablemente compartidas por todos los organismos. En un primer paso la γ-glutamilcisteína (γEC) es sintetizada a partir de Glu y Cys (5), en una reacción dependiente de ATP catalizada por la γ-glutamilcisteína sintetasa (γECS; EC 6.3.3.2). *In vitro* el enzima presenta una alta afinidad por la Cys, mientras que su afinidad por el Glu es mucho menor. Sin embargo, esta circunstancia está compensada por las altas concentraciones de Glu y bajas concentraciones de Cys habitualmente presentes en las células vegetales (Bergmann y Rennenberg, 1993).

$$\text{Glu + Cys + ATP} \xrightarrow{\gamma\text{ECS}} \gamma\text{Glu-Cys + ADP + P}_i \qquad (5)$$

$$\gamma\text{Glu-Cys + Gly + ATP} \xrightarrow{\text{GSHS}} \gamma\text{Glu-Cys-Gly + ADP + Pi} \qquad (6)$$

En el segundo paso (6), también dependiente de ATP, la Gly es añadida al C-terminal de la γEC para formar GSH, en una reacción catalizada por la glutatión sintetasa (GSHS; EC 6.3.2.3). Aunque en las plantas la concentración de γEC es normalmente mucho menor que la concentración de Gly, *in vitro* la afinidad del enzima por ambos sustratos es similar. Esto sugiere que la disponibilidad de γEC puede regular *in vivo* la de síntesis de GSH (Bergmann y Rennenberg, 1993).

La presencia de hGSH parece estar determinada por la existencia de una homoglutatión sintetasa (hGSHS), que añadiría βAla al C-terminal de la γEC en lugar de Gly (Macnicol, 1987; Klapheck, 1988; Bergmann y Rennenberg, 1993).

$$\gamma\text{Glu-Cys + }\beta\text{Ala + ATP} \xrightarrow{\text{hGSHS}} \gamma\text{Glu-Cys-}\beta\text{Ala + ADP + Pi}$$

La GSHS de mamíferos (Rathbun y cols., 1977; Oppenheimer y cols., 1979), así como la GSHS de levaduras (Mooz y Meister, 1967) y tabaco (Hell y Bergmann, 1988), no aceptan βAla como sustrato. Sin embargo, Macnicol (1987) purificó a partir de tallos de *Vigna radiata* un enzima con una afinidad por la βAla seis veces mayor que por la Gly, y que por tanto corresponde probablemente a una hGSHS.

1.6.2. Localización de los enzimas implicados en la síntesis de GSH

Los dos enzimas implicados en la síntesis de GSH han sido localizados en los cloroplastos y en el citosol (Bergmann y Rennenberg, 1993). Sin embargo, la contribución de ambos compartimentos celulares a la síntesis total de GSH en las hojas permanece sin dilucidar. Esta distribución puede variar durante el desarrollo de la planta dependiendo de la fuente de Cys. Por ejemplo, en las hojas maduras la Cys es predominantemente sintetizada en los cloroplastos (Giovanelli, 1990), lo cual sugiere que la síntesis de GSH también se produce principalmente en este orgánulo. Sin embargo, en los estadíos iniciales del desarrollo de las hojas, cuando la Cys proviene mayoritariamente de la degradación de proteínas, el GSH parece ser sintetizado en gran medida en el citosol. Los enzimas implicados en la biosíntesis de GSH han sido también localizados en raíces (Rüegsegger y Brunold, 1992), donde la actividad γECS se distribuye por igual entre los proplastidios y el citosol, mientras que la GSHS fue localizada principalmente en el citosol.

En cuanto a la hGSHS, los únicos estudios realizados hasta la fecha muestran una localización similar de la hGSHS en hojas de *Phaseolus coccineus* (Klapheck y cols., 1988) a la de la GSHS en otras plantas.

1.6.3. Regulación de la biosíntesis de GSH

La concentración total de GSH en las células vegetales depende, al menos en parte, de las actividades de los dos enzimas que participan en su ruta biosintética. Sin embargo, debido a sus bajas concentraciones y a su labilidad, ninguno de los dos enzimas ha sido purificado totalmente a partir de tejidos vegetales, por lo que, con la excepción del tabaco (Hell y Bergmann, 1988, 1990), nuestro conocimiento de los procesos implicados en la biosíntesis del GSH son todavía escasos.

Tanto la actividad γECS, que parece ser limitante en la biosíntesis de GSH, como la actividad GSHS están controladas a distintos niveles:

1. Existen evidencias de que en el primer paso de la síntesis de GSH hay una regulación de tipo "feedback" de la γECS por el GSH. *In vitro*, la actividad γECS es inhibida por concentraciones fisiológicas de GSH (Bergmann y Rennenberg, 1993). Este tipo de control permitiría a las plantas responder rápidamente al aumento de las necesidades de GSH, ya que una caída en los niveles de éste daría lugar a un aumento de la biosíntesis al cesar la inhibición sobre la γECS. Por ejemplo, la oxidación del GSH a GSSG bajo condiciones de estrés oxidativo permitiría un aumento de la actividad γECS y por tanto de la biosíntesis de GSH (Smith y cols., 1984). En cualquier caso, la compartimentación del GSH y de los enzimas implicados

en su síntesis en los diferentes orgánulos celulares podría limitar *in vivo* la eficacia del control "feedback" sobre la velocidad de síntesis de GSH.

2. Diversos autores han observado un incremento en los niveles de GSH cuando las plantas son sometidas a diversos estreses ambientales (Hatzios y Hoagland, 1989; Lamoureux y Rusness, 1989; De Kok, 1990; Smith y cols., 1990; Polle y Rennenberg, 1994). Estos hallazgos sugieren que además de la inhibición de la γECS, existen otros mecanismos, probablemente a nivel molecular, de regulación de la biosíntesis de GSH.

3. La disponibilidad de Cys también parece tener un importante papel regulador sobre la actividad γECS (Farago y cols., 1994; Strohm y cols., 1995; Noctor y cols., 1996, 1997). Ya que el GSH constituye una importante reserva de azufre en estado reducido, la biosíntesis de GSH estaría relacionada con la asimilación de azufre y con la formación de Cys (Rennenberg, 1982; Farago y cols., 1994). Se han observado cambios en la concentración de GSH dependiendo de la disponibilidad de azufre, así como una relación inversa entre los niveles de GSH y la actividad de los enzimas que intervienen en la asimilación de azufre (Lappartient y Touraine, 1996, 1997).

4. La formación de GSH puede estar regulada por el aumento de la síntesis *de novo* de los dos enzimas implicados en su ruta biosintética (Chen y Goldsbrough, 1994). Asimismo, datos preliminares apuntan a una regulación a nivel de la transcripción (Schäfer y cols, 1997) y a modificaciones post-traduccionales (May y cols., 1998b) como mecanismos de control que podrían contribuir a la regulación de la actividad γECS.

1.6.4. El papel de la glutatión reductasa

La glutatión reductasa cataliza la reducción dependiente de NADPH del GSSG a dos moléculas de GSH:

$$GSSG + NADPH \longrightarrow 2\,GSH + NADP^{+}$$

La glutatión reductasa es un enzima ubicuo en bacterias, hongos, protozoos, animales y plantas, debido a que el mantenimiento de una relación GSH/GSSG elevada resulta fundamental en el control del estado redox de prácticamente todas las células (Smith y cols., 1989).

1.6.5. Funciones del GSH

Función antioxidante

El GSH elimina por reacción directa el radical ·OH, altamente tóxico (Foyer, 1984; Halliwell y Gutteridge, 1999), así como los radicales orgánicos (equilibrio 7-8) y el O_2^- (Halliwell y Gutteridge, 1999). La función del GSH como antioxidante se hace más evidente bajo condiciones de estrés, donde son inducidas la síntesis de GSH y la actividad glutatión reductasa (Alscher 1989; Smith y cols., 1989).

$$R\cdot + GSH \longrightarrow RH + GS\cdot \qquad (7)$$

$$2\,GS\cdot \longrightarrow GSSG \qquad (8)$$

Además, el GSH participa en la reducción del deshidroascorbato a ascorbato, tanto de forma directa como en la reacción catalizada por la deshidroascorbato reductasa.

$$2\,GSH + \text{deshidroascorbato} \longrightarrow GSSG + \text{ascorbato}$$

A valores de pH en torno a 8, similares a los existentes en el estroma de los cloroplastos iluminados, la reducción directa del deshidroascorbato puede ser significativa (Foyer, 1984). Sin embargo, la presencia en los cloroplastos de la deshidroascorbato reductasa facilita enormemente la reacción.

En las plantas la función como antioxidante mejor documentada del GSH es su participación en la eliminación del H_2O_2 en los cloroplastos. El H_2O_2 es producido en la reacción Mehler y como resultado de la actividad SOD, y es eliminado a través de una serie de reacciones, conocidas como ciclo ascorbato-GSH, que ya se describieron anteriormente. Todos los enzimas participantes en el ciclo ascorbato-GSH se encuentran en los cloroplastos y en el citosol de las células de las hojas (Gillham y Dodge, 1986), así como en tejidos no fotosintéticos tales como los nódulos de leguminosas (Dalton y cols., 1986).

También se ha descrito la participación del hGSH en la eliminación del H_2O_2 (Zopes y cols., 1993).

Transporte y almacenamiento de azufre

En las plantas superiores el GSH puede ser transportado de unas zonas a otras de la planta a través del xilema y del floema (Garsed y Read, 1977 a,b; Rennenberg y

cols., 1979; Bonas y cols., 1982; Rennenberg y Thoene, 1987; Schupp y cols., 1992). Esto sugiere que el GSH desempeña un importante papel en la distribución del azufre reducido y en consecuencia en el metabolismo del azufre. En varias especies el GSH es transportado desde las hojas maduras (Rennenberg y cols., 1979; Bonas y cols., 1982; Rennenberg y Thoene, 1987) o las semillas (Rauser y cols., 1991) a las raíces, así como desde las hojas maduras a las inmaduras (Rennenberg y cols., 1979; Bonas y cols., 1982; Rennenberg y Thoene, 1987; Schupp y Rennenberg, 1992), siendo incorporado en las proteínas de las hojas más jóvenes (Schupp y cols., 1992). Asimismo, el hGSH es también una molécula transportadora de azufre reducido en plantas de *Vigna radiata* (Macnicol y Bergmann, 1984).

Resistencia frente a metales pesados y xenobióticos

En las plantas y algunos hongos los metales pesados, principalmente el Cd, aunque también Cu, Zn, Pb y Ni, inducen la síntesis de péptidos de estructura general $(\gamma Glu\text{-}Cys)_nGly$ (n= 2-11) conocidos como fitoquelatinas (Rauser, 1990; Zenk, 1996). Estos péptidos forman complejos con los metales pesados, siendo finalmente almacenados en las vacuolas de forma no tóxica para las células.

El GSH actúa como precursor de las fitoquelatinas. Así, se han observado descensos en los niveles de GSH tras la inducción de la síntesis de fitoquelatinas por metales pesados en raíces (Rüegsegger y cols., 1990; Tukendorf y Rauser, 1990; Rauser y cols., 1991) y en cultivos celulares (Grill y cols., 1987; Scheller y cols., 1987; Delhaize y cols., 1989; Schneider y Bergmann, 1995). En cultivos celulares de *Silene cucubalus* ha sido descrita una fitoquelatina sintasa, que cataliza la transpeptidación de γEC proveniente de una molécula de GSH a otra molécula de GSH o a la cadena en crecimiento de una fitoquelatina (Grill y cols., 1989; Loeffler y cols., 1989). En las plantas que contienen hGSH aparecen las homofitoquelatinas, de estructura general $(\gamma Glu\text{-}Cys)_n\beta Ala$ (Grill y cols., 1986), aunque el modo en que son sintetizadas permanece sin dilucidar.

El GSH tiene además un importante papel en la destoxificación de xenobióticos, tales como algunos herbicidas. Las glutatión transferasas contituyen una familia de enzimas que catalizan la conjugación del GSH con xenobióticos electrofílicos (Wilce y Parker, 1994). Al igual que en los animales, las glutatión transferasas parecen estar ampliamente distribuidas en las plantas, donde se cree participan en la defensa frente a estreses bióticos y abióticos (Zhou y Goldsbrough, 1993; Hahn y Strittmatter, 1994; Droog y cols., 1995; Ulmasov y cols., 1995).

Regulación de la respuesta de las plantas en condiciones de estrés

El GSH también está implicado en otras funciones muy importantes, relacionadas con la regulación de la respuesta antioxidante de las plantas en condiciones de estrés. Así, se ha propuesto que tanto el nivel de GSH como su estado redox son elementos clave en la respuesta adaptativa de las plantas a los cambios ambientales (May y cols., 1998a).

El GSH induce la expresión de genes de defensa tras el ataque de patógenos (Wingate y cols., 1988; Edwards y cols., 1991; Noctor y Foyer, 1998). Asimismo, tanto el GSH como el GSSG podrían actuar como moléculas señal en condiciones de estrés biótico (Foyer y cols., 1997). Además, se han identificado elementos que responden a GSH en los promotores de genes que codifican glutatión transferasas y en los de genes implicados en la síntesis de fitoalexinas (Dron y cols., 1988; Levine y cols., 1994).

2. *Objetivos*

Los objetivos generales propuestos en esta Tesis han sido:

1. Poner a punto una técnica de HPLC que nos permita determinar con elevada sensibilidad y especificidad los tioles presentes en nódulos, raíces y hojas de leguminosas, así como las correspondientes actividades tiol sintetasa. La técnica deberá ser lo suficientemente precisa como para cuantificar concentraciones picomolar de tioles (especialmente Cys y γEC) en muestras de tejido provenientes de la disección de nódulos.

2. Analizar de forma detallada el metabolismo del GSH y hGSH en nódulos de leguminosas, tanto a nivel bioquímico como molecular, y determinar el papel de los nódulos en la síntesis de tioles en la planta. Para ello se medirán las actividades de los enzimas implicados en la síntesis de ambos tioles.

3. Aislar clones conteniendo cDNAs que codifican los enzimas γECS, GSHS y hGSHS. Las secuencias se utilizarán para hacer construcciones destinadas a modular los niveles de los enzimas mediante la transformación de la leguminosa modelo *Lotus japonicus,* con el fin de estudiar la regulación de la biosíntesis de tioles en los nódulos.

4. Determinar la localización subcelular de los enzimas implicados en la síntesis del GSH y hGSH. Mediante técnicas de fraccionamiento subcelular en gradientes de densidad se aislarán bacteroides, cloroplastos, mitocondrias, peroxisomas y plastidios, y se medirán las diferentes actividades enzimáticas en cada una de las fracciones. Asimismo, a partir de las secuencias de cDNA se harán estudios de predicción de la localización subcelular de cada uno de los enzimas.

5. Estudiar el efecto de la senescencia natural (envejecimiento) e inducida por estrés en la concentración de tioles y en sus rutas biosintéticas. En concreto, se analizarán dos tipos de estrés inducido en los nódulos: exposición prolongada de las plantas a la oscuridad y tratamiento con exceso de nitrato.

6. Estudiar el metabolismo de tioles a nivel tisular en nódulos determinados e indeterminados, ya que ambos tipos de nódulos presentan importantes diferencias estructurales y metabólicas. Los nódulos determinados se diseccionarán en córtex y zona infectada, y los nódulos indeterminados en zonas meristemática, fijadora y senescente.

3. *Glutathione and homoglutathione synthesis in legume root nodules*

3.1. Introduction

The tripeptide GSH is the major non-protein thiol in most animals, plants, and prokaryotes (Meister and Anderson, 1983; Hausladen and Alscher, 1993; Rennenberg, 1997). In plants, GSH is a versatile antioxidant that can directly scavenge activated oxygen species and participate in the ascorbate-GSH cycle for peroxide removal in the chloroplasts. It is also involved in many other vital functions of plants, including the transport and storage of sulfur, the synthesis of proteins and DNA, tolerance to abiotic and biotic stress, and the detoxification of xenobiotics, air pollutants, and heavy metals (Hausladen and Alscher, 1993; Rennenberg, 1997; May et al., 1998a).

The pathway for GSH synthesis is probably shared by all organisms and involves two ATP-dependent steps. In the first reaction γEC is formed from Glu and Cys by γECS, and in the second reaction Gly is added to the C-terminal site of γEC by GSHS. In plants γECS and GSHS are present in the chloroplasts and cytosol of leaves (Law and Halliwell, 1986; Klapheck et al., 1987; Hell and Bergmann, 1988, 1990). More recently, the two enzymes have been found also in the roots of maize (Rüegsegger and Brunold, 1993) and of the heavy metal-accumulator *Brassica juncea* (Schäfer et al., 1998).

Legumes are an interesting plant material with which to study thiol metabolism for various reasons. First, there is an active ascorbate-GSH cycle in the root nodules, which requires a continuous supply of GSH to protect N_2 fixation against toxic oxygen species (Dalton et al., 1986). Second, the leaves, roots, and seeds of some legumes contain a thiol tripeptide homolog (hGSH), instead of or in addition to GSH. The synthesis of hGSH is thought to proceed through γECS and a specific hGSHS (Macnicol, 1987; Klapheck, 1988). Third, GSH is believed to be involved in plant morphogenesis, cell division, control of redox status, and signaling

of stress and pathogen attack (Wingate et al., 1988; May et al., 1998a). All these processes, with some modifications (Vasse et al., 1990; Hirsch, 1992; Baron and Zambryski, 1995), are important in nodule formation and functioning, and therefore GSH is likely to be a critical molecule of nodules.

There is scant information about thiol compounds of legume nodules. Thiol tripeptides are known to be at high concentrations in nodules (Dalton et al., 1991; Escuredo et al., 1996; Gogorcena et al., 1995, 1997), but this information is based on an enzymatic assay that does not distinguish between GSH and hGSH (Griffith, 1980). Very recently, Evans et al. (1999) reported that hGSH is more abundant than GSH in soybean nodules. However, they employed an HPLC technique based on the formation and UV detection of dinitrophenyl derivatives from the reaction of 1-fluoro-2,4-dinitrobenzene with the amino groups (Farris and Reed, 1987). The technique is slow since it requires over-night derivatization and lacks the necessary sensitivity and specificity to quantify thiols in small nodule samples or dissected nodule fractions. This is especially true for Cys and γEC, which are present in plant tissues at low concentrations and are also essential for the study of thiol metabolism. Evans et al. (1999) also concluded that natural senescence in soybean nodules is an oxidative stress process. They reported, for example, a decrease in thiol content and increases in catalytic iron, thiol oxidation, and oxidative damage. A few years earlier we reached the same conclusions about stress-induced nodule senescence (Escuredo et al., 1996; Gogorcena et al., 1995, 1997).

The latest paper within this extensive study on stress-induced nodule senescence (Matamoros et al., 1999a) reported that thiol contents and thiol synthetase activities of nodules could be conveniently assayed using HPLC with fluorescence detection. In the present study, we have improved this methodology and examined in detail thiol metabolism in legume nodules. Our results show that nodules are a main site of GSH and hGSH synthesis within the plant and provide indirect evidence that thiol compounds play a crucial role in the process of N_2 fixation.

3.2. Materials and methods

3.2.1. Plant and bacterial material

The legume-rhizobia symbioses used in this study are indicated in Table 3. Nodulated plants were grown in pots containing a 2:1 (v/v) perlite:vermiculite mixture with nitrogen-free nutrient solution under controlled environmental conditions (Gogorcena et al., 1997). For senescence studies, the age, growth stage,

and treatments of plants are indicated in Tables 7 and 8. For other experiments, all legumes were between 30 and 35-d old when harvested, except alfalfa, which was between 50 and 54-d old. All plants were at the vigorous vegetative growth stage. Nodules for dissection studies were processed immediately after harvest. All other plant material was flash-frozen in liquid nitrogen and stored at -80°C until extraction.

Table 3. *Plant and bacterial material used in this study*

Common name	Symbiosis
Pea	*Pisum sativum* L. cv Lincoln x *Rhizobium leguminosarum* biovar *viciae* NLV8
Broad bean	*Vicia faba* L. cv Muchamiel x *R. leguminosarum* biovar *viciae* NLV8
Alfalfa	*Medicago sativa* L. cv Aragón x *Sinorhizobium melioti* 102F78
Lupine	*Lupinus albus* L. cv Multolupa x *Bradyrhizobium* sp. (*Lupinus*) ISLU16
Soybean	*Glycine max* Merr. cv Williams x *Bradyrhizobium japonicum* USDA110
Bean	*Phaseolus vulgaris* L. cv Contender x *R. leguminosarum* biovar *phaseoli* 3622
Mungbean	*Vigna radiata* Wilczek x *Bradyrhizobium* sp. (*Vigna*) 32H1
Cowpea	*Vigna unguiculata* Walp. cv California # 5 x *Bradyrhizobium* sp. (*Vigna)* 32H1

3.2.2. Thiol analysis

Extraction and analysis of thiol compounds were performed by modifying earlier procedures based on the derivatization of thiols with monobromobimane (MBB) and separation of the highly fluorescent adducts by HPLC (Fahey and Newton, 1987; Klapheck, 1988). For senescence and dissection studies, 10 to 20 mg of whole nodules or dissected nodule tissue was used. Although assays could be performed with 10 mg or even lower amounts of plant tissue, 50 mg of nodules and

250 mg of leaves or roots were employed when material was not limiting. Volumes of extraction medium and derivatization solution were adjusted accordingly.

Nodules (50 mg) were ground at 0°C in an Eppendorf tube with 500 µL of 200 mM methanesulfonic acid (containing 0.5 mM diethylenetriaminepentaacetic acid). The homogenate was centrifuged at 13,000*g* for 5 min in the cold and 200 µL of sample was mixed with 92 µL of 8 mM dithioerythritol (DTE), 400 µL of 200 mM N-[2-hydroxyethyl]piperazine-N'-3-propanesulfonic acid (EPPS) buffer, pH 8.0 (containing 5 mM diethylenetriaminepentaacetic acid), and 8 µL of 5 M NaOH. After incubation for 1 h at room temperature, 200 µL of 7 mM MBB (Calbiochem) was added, and the mix was further incubated for 15 min in the dark. The reaction was stopped by the addition of 350 µL of 20% (v/v) acetic acid. Samples were stored at -80°C for several days before analysis. The samples were centrifuged and filtered, and 10-µL aliquots were injected on the HPLC. The MBB derivatives were resolved on a Waters Nova-Pak C_{18} column (3.9 x 150 mm; 4 µm, Nova-Pak, Waters, Milford, MA), eluted with 15% methanol/0.25% (v/v) acetic acid (pH 3.5) at 1 mL min^{-1}, and detected by fluorescence (model 474 detector; Waters) with excitation at 380 nm and emission at 480 nm. The proportion of oxidized glutathione was determined in nodule extracts prepared as before using glutathione reductase and 2-vinylpyridine (Griffith, 1980). For both HPLC and enzymatic thiol determinations, stock solutions of Cys, γEC, GSH, and hGSH were titrated with the Ellman's reagent using an extinction coefficient for 2-nitro-5-thiobenzoate of 13.6 mM^{-1} cm^{-1} at 412 nm (Ellman, 1959).

3.2.3. Thiol synthetase assays

Extraction and assays of γECS, GSHS, and hGSHS were performed by modification of previous methods (Hell and Bergmann, 1988; Kocsy et al., 1996). Some of these modifications were critical to measure thiol synthetase activities, especially γECS, in nodules. All activities were measured within linear range.

Nodules (50 mg) were ground in an Eppendorf tube with 500 µL of 50 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 10% glycerol, and 10 mM $MgCl_2$. The homogenate was centrifuged at 13,000*g* for 10 min and the supernatant was freed from small molecules by repeated dilution and concentration over ultrafiltration membranes (Centricon-10; Amicon, Beverly, MA). The activity of γECS was assayed by HPLC quantification of the synthesized γEC as its MBB derivative. The reaction mixture contained 120 mM HEPES (pH 8.0), 60 mM $MgCl_2$, 6 mM ATP, 6 mM PEP, 6 units of pyruvate kinase, 0.5 mM DTE, 48 mM L-Glu, and 100 µL of extract, in a total volume

of 235 µL. The reaction was initiated by the addition of 15 µL of 40 mM L-Cys and terminated after 0 and 60 min at 30°C by transferring an aliquot of 80 µL into derivatization solution. This consisted of 300 µL of 200 mM EPPS buffer, pH 8.0 (containing 5 mM diethylenetriaminepentaacetic acid), and 120 µL of 7 mM MBB. Derivatization was carried out for 15 min at room temperature in the dark and stopped by the addition of 97 µL of 40% (v/v) acetic acid. Samples were stored at -80°C for subsequent HPLC analysis as before. The activities of GSHS and hGSHS were assayed by HPLC quantification of the synthesized GSH or hGSH as their MBB derivatives. The reaction mixture contained 125 mM Tris-HCl (pH 8.5), 50 mM KCl, 25 mM $MgCl_2$, 5 mM ATP, 5 mM PEP, 5 units of pyruvate kinase, 5 mM DTE, 0.5 mM γEC, and 5 mM Gly (GSHS) or βAla (hGSHS), in a total volume of 100 µL. After preincubation at 30°C for 3 min, the reaction was initiated by adding 100 µL of sample and stopped after 0 and 60 min at 30°C by derivatization of aliquots, as described above.

3.2.4. Isolation of complete cDNA sequences encoding γECS from bean and pea nodules

Primers (sense: 5'-GAGCTTAGTGGTGCACC[A/T]CTTGA-3' and antisense: 5'-TGCTCAAACCCAAAAGAGTCAT-3') were designed to conserved γECS cDNA sequences of tomato, *B. juncea*, and Arabidopsis. Bean and pea nodule cDNA Lambda ZAP libraries (generously provided by Dr. Carroll Vance, U.S. Deparment of Agriculture-University of Minnesota, St. Paul) were used as templates for *γecs* internal sequence amplification. PCR components and concentrations were as follows: 1 µM for sense primer and 0.2 µM for antisense primer, 200 µM for each dNTP, 2.5 mM $MgCl_2$, 0.05% W-1 detergent (GIBCO-BRL, Paisley, UK), and 1.25 units of native *Taq* DNA polymerase (GIBCO-BRL), in a final volume of 25 µL of the PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl). Tubes were pre-incubated at 95°C for 3 min to ensure complete denaturation of DNA. Amplification was carried out for 40 cycles at 55°C for 1 min (annealing), 72°C for 1 min (extension), and 95°C for 1 min (denaturation). Additional annealing and extension steps were done at 55°C for 1 min and 72°C for 11 min, respectively. The total volume of the PCR samples was electrophoresed in agarose gels. The PCR products were extracted using a gel-extraction system (Concert Matrix, GIBCO-BRL) and resuspended in 10 µL of sterile water.

For the PCR isolation of the 5' ends of the γECS cDNAs, the same antisense primer (1 µM) was used with T3 as the sense primer (0.2 µM). For isolation of the 3´ ends, a sense primer (5'-GCTGAGGA[A/G]ATGGGAATTGG-3') and a T7 antisense

primer (both at 0.5 μM) were used. The same PCR program was followed except that the extension steps were at 72°C for 1.5 min and that the annealing step for the amplification of the 3' end fragments was at 58 °C for 1 min. PCR products were gel purified as indicated above.

Aliquots of the resuspended DNA were used to clone each PCR product into the linearized vectors pGEM-T (Promega, Madison, WI) or pCR2.1 (Invitrogen, Carlsbad, CA) following the procedures supplied by the manufacturers. The relevant cDNA clones were sequenced in both directions by the dideoxy method (Sanger et al., 1977) using an automated sequencer (PRISM 377, Applied Biosystems, Foster City, CA). Database searches were performed at the National Center for Biotechnology Information by using the BLAST network service. Sequence analyses were done using the Genetics Computer Group (Madison, WI) package.

3.2.5. Dissection studies

Fresh bean and pea nodules were dissected under the binocular microscope with a sharp surgery blade. Pieces of nodules (10 mg for thiols and γECS; 20 mg for GSHS and hGSHS) were collected in ice-cold Eppendorf tubes (previously weighed to ± 0.1 mg) and were extracted as described above. The terminology of nodule anatomy described by Vasse et al. (1990) and Hirsch (1992) was followed. Pea nodules were dissected into meristem plus early symbiotic zone (I plus II), late symbiotic zone (III), and senescent zone (IV). Bean nodules were dissected into cortex and infected zone.

3.2.6. Statistical analysis

Two to four series of plants were grown at different dates under identical environment conditions. Samples from each series of plants (six to ten plants per series) were pooled and a similar number of samples were randomly selected from each series. Data of the various series were then pooled for statistical analysis. The number of samples used for calculation of the mean and SE are stated in each table or figure.

3.3. Results

3.3.1. Thiol metabolism of nodules can be reliably studied by HPLC with fluorescence detection

Central to this study was the development of a technique for the sensitive and precise determination of thiols and thiol synthetase activities in small amounts of nodule tissue. Samples were incubated with DTE prior to thiol derivatization with MBB at pH 8.0. This was essential to quantify Cys and γEC, since the two thiols tend to rapidly oxidize in the extracts. Preincubation with DTE also reduced the small quantities of the disulfide forms of GSH and hGSH present in the extracts. Tripeptide disulfides were measured using an enzymatic method (Griffith, 1980) and accounted for 3 to 10% of the total glutathione pool, which is consistent with earlier reports showing that most glutathione in plant cells is in the reduced form (for review, see Hausladen and Alscher, 1993).

The measurement of thiol synthetase activities, especially γECS, in whole or fractionated nodules involved more serious difficulties. First, it was essential to deplete extracts of endogenous free thiols, Gly, and βAla, which interfered with the assays. This was accomplished efficiently with ultrafiltration membranes. The process could be completed within 2 h, whereas conventional dialysis required overnight incubation, and gel filtration was not accurate enough with the small volumes (100 to 250 μL) of extracts used in this study. Second, the concentration of DTE was critical for the synthetase assays. Concentrations in the range of 2 to 5 mM have been reported to be inhibitory for γECS from several plants (Hell and Bergmann, 1990). We found that a low DTE concentration (0.48 mM) was not inhibitory but protective for the enzyme, and that a high Cys concentration (2.4 mM) was optimal to assay for γECS activity. This was because Cys was rapidly oxidized by plant extracts, and lower concentrations of this substrate could become nonsaturating for γECS in the course of the assay.

Additional controls for the assay of γECS activity included boiling of extracts (30 min), omission of ATP or Cys, and preincubation with buthionine sulfoximine (10 mM, 10 min, 30°C), a specific inhibitor of γECS in the presence of ATP (Huang et al., 1988). Additional controls for the assay of GSHS and hGSHS activities included boiling of extracts (30 min) and omission of ATP or γEC. As expected, no activity could be detected under any of those conditions.

Using the modified technique described in this work, samples of 10 mg or lower were readily analyzed for thiol content and synthetase activities. The method permitted the peak-base separation of Cys, γEC, GSH, and hGSH in a single run with isocratic elution (Fig. 7). The corresponding MBB derivatives showed retention times of approximately 3, 4, 5, and 9 min, and their fluorescence response was linear for at least up to 16 pmol for Cys and γEC and 160 pmol for GSH and hGSH. Samples

containing as little as 2 pmol of thiols per 10-µL injection could be accurately measured. Sensitivity can be further enhanced by a factor of two or three by changing the volumes of injection and of the derivatization mixture, but it is at least 50-fold greater than that reported by Farris and Reed (1987).

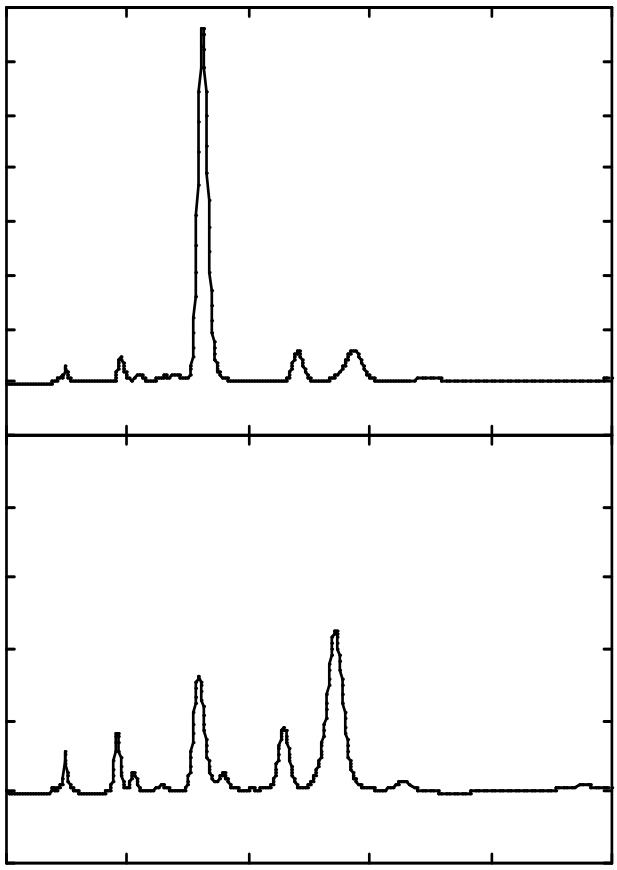


Figure 7. *Representative HPLC analysis of thiol compounds in legume nodules. A, Soluble extract of pea nodules showing GSH as the predominant thiol. B, Soluble extract of bean nodules showing hGSH as the predominant thiol. Peaks of Cys and γEC are also labeled. Other peaks correspond to other MBB adducts.*

3.3.2. Nodules are a major site of thiol synthesis within the plant

The distribution of nonprotein thiols in several legumes of agronomic relevance is indicated in Tables 4 and 5. Four of them (pea, broad bean, alfalfa, and lupine) produce indeterminate nodules (persistent meristems, amide exporters) and the other four (soybean, bean, cowpea, and mungbean) produce determinate nodules

(no persistent meristems, ureide exporters). The two types of nodules show important structural and metabolic differences (Hirsch, 1992) and hence a comparison was of considerable interest.

Legume nodules contained, on average, 6-fold more thiol tripeptides (GSH plus hGSH) than the roots and 2.2-fold more than the leaves of the same plants. The relative abundance of GSH and hGSH was strikingly dependent on the legume species and plant tissue. All indeterminate nodules examined contained GSH as the major or sole tripeptide. This was also true for the corresponding leaves, with the exception of alfalfa. In this legume, GSH was the most abundant (72%) tripeptide in nodules, but, surprisingly, hGSH predominated by far in leaves (98%) and roots (82%). On the other hand, hGSH was the most abundant tripeptide in the determinate nodules of soybean, bean, and mungbean. These legumes were previously described as containing almost exclusively hGSH in leaves, roots, and seeds (Klapheck, 1988). Our data confirm that hGSH was virtually the only tripeptide (>98%) present in the leaves and roots of those legumes, but show, that their determinate nodules contained substantial amounts (22%-38%) of GSH (Table 4). Unexpectedly, the leaves and determinate nodules of cowpea *(Vigna unguiculata),* a species closely related to mungbean *(Vigna radiata),* contained almost exclusively GSH. Clearly, the production of hGSH is not a characteristic feature of the tribe Phaseoleae, contrary to some early suggestions (Grill et al., 1986; Klapheck, 1988), nor is it linked to specific structural or metabolic features of determinate nodules, such as ureide production.

Table 4. *GSH and hGSH content in legume nodules, roots and leaves. Data are means ± SE of four to eight samples. N, nodules; R, roots; L, leaves. Thiol contents are expressed in nanomoles per gram fresh weight.*

Legume	GSH			hGSH		
	N	R	L	N	R	L
Pea	829 ± 71	147 ± 15	445 ± 18	107 ± 10	69 ± 11	0
Broad bean	650 ± 9	96 ± 1	383 ± 27	0	0	0
Alfalfa	840 ± 53	36 ± 3	16 ± 3	327 ± 31	166 ± 14	887 ± 22
Lupine	340 ± 33	49 ± 3	120 ± 32	0	0	0
Soybean	130 ± 7	3 ± 3	7 ± 4	471 ± 48	235 ± 38	305 ± 23
Bean	119 ± 10	1 ± 1	0	322 ± 14	70 ± 6	198 ± 24
Mungbean	217 ± 11	4 ± 2	1 ± 1	356 ± 30	74 ± 13	170 ± 38
Cowpea	587 ± 28	35 ± 4	270 ± 25	7 ± 2	37 ± 3	0

The thiol precursors Cys and γEC were more abundant in nodules than in leaves or roots, but were present at much lower levels (<15%) than the total tripeptides in all tissues examined (Table 5). The content of Cys was greater than that of γEC for all legume tissues except in alfalfa and lupine nodules. In fact, the dipeptide was not detectable in the roots or leaves of most legumes. Considering a 85% water content, the average concentrations of thiols in nodules can be roughly estimated in the range of 30 to 120 μM for Cys, 7 to 50 μM for γEC, and 0.4 to 1.4 mM for total thiol tripeptides.

Table 5. *Cys and γEC content in legume nodules, roots and leaves. Data are means ± SE of four to eight samples. N, nodules; R, roots; L, leaves. Thiol contents are expressed in nanomoles per gram fresh weight.*

Legume	Cys			γEC [a]	
	N	R	L	N	L
Pea	55 ± 5	12 ± 2	4 ± 3	15 ± 4	2 ± 2
Broad bean	25 ± 1	1 ± 1	11 ± 0	6 ± 0	0
Alfalfa	90 ± 2	20 ± 2	17 ± 1	95 ± 3	3 ± 2
Lupine	12 ± 2	0	23 ± 2	37 ± 5	8 ± 3
Soybean	52 ± 3	11 ± 1	13 ± 3	5 ± 1	5 ± 3
Bean	39 ± 2	11 ± 1	13 ± 2	4 ± 2	8 ± 4
Mungbean	74 ± 6	6 ± 2	12 ± 2	24 ± 2	3 ± 1
Cowpea	99 ± 15	0	23 ± 5	8 ± 3	0

[a] There was no detectable γEC in the roots for any legume species

The finding that nodules have substantially greater levels of GSH, hGSH, and their thiol precursors than the leaves, which are considered a main source of non-protein thiols within the plant (Rennenberg, 1997), is a strong indication that GSH and hGSH are synthesized in nodules. This was confirmed by the determination of all of the enzyme activities required for the synthesis of thiol tripeptides in nodule extracts (Table 6). By using optimized methods, γECS activity, traditionally recalcitrant to assay because of the low level and instability of the enzyme, was clearly measurable at rates between 2 and 9 nmol γEC min^{-1} g^{-1} fresh weight. Similarly, GSHS was found to be the predominant (pea, alfalfa) or exclusive (broad bean, lupine) thiol tripeptide synthetase in indeterminate nodules, and hGSHS was the predominant (soybean, bean) or exclusive (mungbean) synthetase in determinate

nodules, with the exception of cowpea (Table 6). Thus, only GSHS was detected in cowpea nodules and, in fact, at greater activity rates than in the other legumes (Table 6). This observation is fully consistent with GSH being the only tripeptide present in cowpea nodules (Table 4).

Table 6. *Enzyme activities involved in GSH and hGSH synthesis in legume nodules. Data are means ± SE of four to six samples. Enzyme activities are expressed in nanomoles per minute per gram fresh weight.*

Legume	γECS	GSHS	hGSHS
Pea	6.6 ± 1.2	11.4 ± 1.2	6.9 ± 0.4
Broad bean	8.3 ± 1.1	8.2 ± 0.7	0
Alfalfa	3.3 ± 1.0	4.4 ± 0.5	1.1 ± 0.3
Lupine	9.4 ± 1.3	14.9 ± 1.6	0
Soybean	1.7 ± 0.2	2.6 ± 0.9	6.3 ± 0.2
Bean	4.5 ± 1.3	0.7 ± 0.2	7.0 ± 1.0
Mungbean	2.7 ± 0.8	0	6.0 ± 1.0
Cowpea	3.0 ± 1.5	15.8 ± 1.8	0

3.3.3. Thiol content and synthesis decrease with nodule senescence

To study the effect of natural (aging) and stress-induced nodule senescence on thiol composition and synthesis, samples of indeterminate (pea) and determinate (bean) nodules were harvested at fixed time points during plant ontogeny. In both pea (Table 7) and bean (Table 8), there was a steady decline of non-protein thiols with advancing age. As could be anticipated, this was accompanied by the loss of nodule soluble protein and an increase in the shoot fresh weight, marking, respectively, the progression of nodule senescence and the transition from the vegetative stage to the flowering and fruiting stages of plants.

Mature pea nodules (5 weeks) contained between 25% and 45% less protein, Cys, thiol tripeptides, and thiol synthetase activities than young nodules (3 weeks). The reductions in these parameters were between 55% and 82% for old nodules (7 weeks). The dipeptide γEC was only detectable (and at low levels) in young nodules (Table 7). Placement of young pea plants for 4 d in continuous darkness induced nodule senescence, as was clearly evidenced by a 36% decline in the total soluble protein of nodules. Dark treatment led to decreases in thiols and thiol synthetase activities, with the exception of γECS, down to the values recorded for mature and old nodules. It is noteworthy that the dark treatment caused a 92% decline in the

content of GSH (the major tripeptide) of young nodules, but only a 38% decrease in hGSH content and a 79% increase in the extractable γECS activity (Table 7).

Table 7. *Effect of aging and stress-induced senescence on thiol composition and synthesis in pea nodules. Data are means ± SE of three to six samples. Protein is expressed in milligrams per gram fresh weight; shoot fresh weight is expressed in gram per plant; thiol contents are expressed in nanomoles per gram fresh weight; enzyme activities are expressed in nanomoles per minute per gram fresh weight.*

Parameter	3 [a]	5 [b]	7 [c]	3 + D [d]
Protein	22.8 ± 0.9	12.2 ± 0.9	8.4 ± 0.8	14.7 ± 2.4
Shoot fresh wt	2.1 ± 0.3	5.3 ± 1.2	9.6 ± 0.7	1.8 ± 0.3
Cys	61.0 ± 4.2	39.7 ± 8.4	27.5 ± 1.9	26.1 ± 2.5
γEC	3.7 ± 0.4	0.8 ± 0.6	0	0
GSH1267 ± 37	868 ± 91	224 ± 52	102 ± 24	
hGSH	138 ± 11	104 ± 14	49 ± 6	85 ± 16
γECS	4.2 ± 0.4	2.6 ± 0.8	1.4 ± 0.1	7.5 ± 0.7
GSHS	17.2 ± 0.6	12.4 ± 2.5	6.0 ± 1.1	8.3 ± 1.6
hGSHS	10.0 ± 1.0	7.1 ± 0.7	2.9 ± 0.9	4.9 ± 0.4

[a] Nodule age, 3 weeks; plant age, 27 d; phenological stage, early vegetative. [b] Nodule age, 5 weeks; plant age 41 d; phenological stage, late vegetative. [c] Nodule age, 7 weeks; plant age, 55 d; phenological stage, late flowering-early fruiting. [d] Nodule age, 3 weeks; plant age, 27 d (plus 4 d of treatment); treatment, 4 d in continuous darkness; phenological stage, early vegetative.

Similarly, mature bean nodules (5 weeks) had between 20% and 40% less protein, Cys, thiol tripeptides, and hGSHS activity than young nodules (3 weeks), 84% less γECS activity, and no detectable GSHS activity (Table 8). In old nodules (7 weeks), the contents of Cys and thiol tripeptides were 30% to 45% lower than those of young nodules, whereas soluble protein was reduced by 65% and γECS and hGSHS activities by 80%. Induction of bean nodule senescence by nitrate, as evidenced by a 44% decline in soluble protein, caused decreases of 50% to 60% in Cys, γECS activity, and hGSHS activity down to or below the values observed in the oldest nodules (Table 8). The nitrate-induced declines in the nodule contents of hGSH (the major tripeptide) and GSH were 89% and 59%, respectively, but the age-related decline was approximately 35% for both thiols (Table 8).

Table 8. *Effect of aging and stress-induced senescence on thiol composition and synthesis in bean nodules. Data are means ± SE of three to six samples. Protein is expressed in milligrams per gram fresh weight; shoot fresh weight is expressed in gram per plant; thiol contents are expressed in nanomoles per gram fresh weight; enzyme activities are expressed in nanomoles per minute per gram fresh weight.*

Parameter	3 [a]	5 [b]	7 [c]	3 + N [d]
Protein	17.2 ± 1.7	13.9 ± 0.2	6.1 ± 1.7	9.6 ± 1.3
Shoot fresh wt	5.7 ± 0.4	22.7 ± 2.0	50.3 ± 4.5	5.4 ± 1.1
Cys	42.3 ± 1.8	28.7 ± 3.2	23.1 ± 2.8	17.2 ± 2.6
γEC	1.3 ± 0.8	0	0	0
GSH180 ± 18	142 ± 17	126 ± 9	74 ± 10	
hGSH	350 ± 29	204 ± 9	225 ± 12	39 ± 5
γECS	4.5 ± 1.1	0.7 ± 0.1	0.7 ± 0.4	1.9 ± 0.9
GSHS	0.9 ± 0.3	0	0	0.3 ± 0.1
hGSHS	10.6 ± 0.8	7.3 ± 1.4	2.0 ± 0.6	5.2 ± 0.3

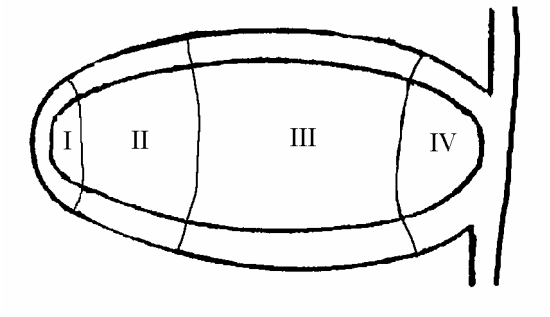
[a] Nodule age, 3 weeks; plant age, 25 d; phenological stage, vegetative. [b] Nodule age, 5 weeks; plant age 39 d; phenological stage, late flowering-early fruiting. [c] Nodule age, 7 weeks; plant age, 53 d; phenological stage, fruiting. [d] Nodule age, 3 weeks; plant age, 25 d (plus 4 d of treatment); treatment, 4 d with nitrate; phenological stage, early vegetative.

3.3.4. Thiol synthesis is especially active in the meristematic and infected zones of pea nodules

Experiments were designed to further investigate the effect of age at the tissue level and the possible association between thiol synthesis and N_2 fixation. Indeterminate nodules characteristically show an age gradient from the apical meristem to the senescent basal tissue (Vasse et al., 1990; Hirsch, 1992). Pea nodules were dissected into meristematic-early symbiotic zone (white, with no detectable Lb), the late symbiotic zone (bright red), and senescent zone (brown-green, indicative of Lb degradation).

The senescent zone contained 50% less GSH and 25% less Cys and hGSH than the other zones (Fig. 8). However, γECS activity was approximately 4.3 nmol γEC $min^{-1}g^{-1}$ fresh weight in all three zones in which nodules were dissected, indicating that this activity was not limiting GSH or hGSH synthesis in the senescent zone. In contrast, there was a progressive decline in GSHS and hGSHS activities with the age of nodule tissue. The senescent zone had 45% less GSHS activity and 71% less

hGSHS activity than the meristematic-early symbiotic zone, which may explain at least in part the lower GSH and hGSH contents in the former. The estimated concentrations of Cys (0.12 mM), GSH (2.3 mM), and hGSH (0.25 mM) were similar for both the meristematic-early symbiotic zone and the late symbiotic zone.



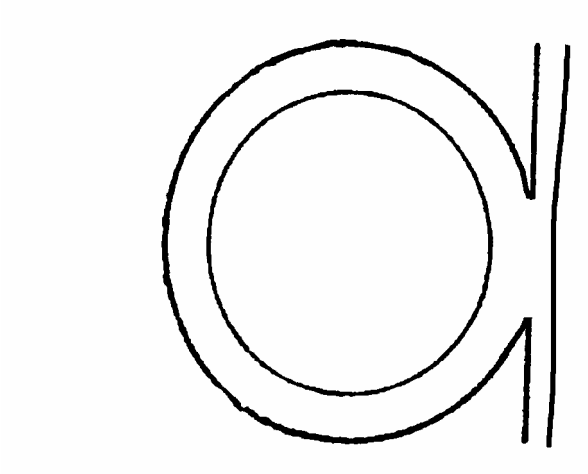
	Zone I+II	Zone III	Zone IV
Cys	100 ± 11	100 ± 6	72 ± 2
γEC	3 ± 0.3	1.9 ± 0.5	0.4 ± 0.2
GSH	1897 ± 81	2018 ± 122	976 ± 58
hGSH	205 ± 14	221 ± 7	161 ± 7
γECS	4.3 ± 0.3	4.2 ± 0.6	4.6 ± 0.4
GSHS	19.1 ± 1.5	15.1 ± 0.1	10.6 ± 1.4
hGSHS	17.4 ± 2.0	8.9 ± 0.5	5.0 ± 0.7

Figure 8. *Thiol composition and synthesis in different zones of pea nodules. Data are means ± SE of four to eight samples of nodule fractions. Thiol contents are expressed in nanomoles per gram fresh weight and enzyme activities in nanomoles per minute per gram fresh weight.*

3.3.5. γECS and hGSHS are more abundant, respectively, in the infected zone and cortex of bean nodules

In determinate nodules, cell divisions cease early during nodule development and there are no obvious age differences among the various nodule tissues (Hirsch, 1992). In this case, bean nodules were dissected into the cortex and infected zone to investigate whether thiol synthesis was more active in the nitrogen-fixing tissue. The infected zone had between 2 and 5-fold more Cys, GSH, hGSH, and γECS activity than the nodule cortex (Fig. 9). The infected zone also contained 12 nmol γEC g^{-1} fresh weight, whereas the dipeptide was virtually below detection levels in the

cortex. The proportion of thiol tripeptides was different in the two nodule tissues, with a hGSH/GSH ratio of 3.8 in the cortex and 1.5 in the infected zone. The estimated concentrations of thiols in the cortex were 26 μM Cys, <1 μM γEC, 65 μM GSH, and 247 μM hGSH. The corresponding concentrations in the infected zone were 122 μM Cys, 14 μM γEC, 281 μM GSH, and 409 μM hGSH.



	Cortex	Infected zone
Cys	22 ± 4	104 ± 7
γEC	1 ± 1	12 ± 3
GSH	55 ± 4	239 ± 21
hGSH	210 ± 17	348 ± 35
γECS	1.9 ± 0.4	4.6 ± 0.4
GSHS	1.4 ± 0.3	1.8 ± 0.1
hGSHS	11.4 ± 1.5	4.9 ± 1.0

Figure 9. *Thiol composition and synthesis in the cortex and infected zone of bean nodules. Data are means ± SE of four to eight samples of nodule fractions. Thiol contents are expressed in nanomoles per gram fresh weight and enzyme activities in nanomoles per minute per gram fresh weight.*

The relative activities of the thiol tripeptide synthetases were also clearly different in the cortex and infected tissue. Whereas both nodule regions showed similar values of GSHS activity, the nodule cortex had 2.3-fold more hGSHS activity than the infected zone or, in other terms, accounted for 70% of the whole nodule hGSHS activity on a fresh weight basis (Fig. 9). This remarkably high hGSHS activity in the cortex was consistently found in all the dissection experiments conducted in this work.

3.3.6. Complete cDNA sequences reveal high homology among γECS proteins of higher plants

The enzyme γECS is considered to be critical in GSH homeostasis in plants (Rennenberg, 1997; May et al., 1998a; Noctor and Foyer, 1998), and therefore we initiated a study of *γecs* in legume nodules. To this purpose, oligonucleotide primers were designed to conserved *γecs* sequences of tomato, *B. juncea*, and Arabidopsis, and used to screen pea and bean nodule cDNA libraries by PCR. Full-length coding sequences of approximately 2 Kb were obtained for the *γecs* of both legumes and showed high identity with other *γecs* sequences available in the database. Pea and bean cDNA sequences were approximately 80% identical to those of tomato, *B. juncea,* and Arabidopsis. The nucleotide sequences of pea, bean, and *Medicago truncatula* showed between 86% and 91% identity.

The open reading frame (ORF) for the γECS of pea nodules was predicted to encode a 499-amino acid polypeptide, with an expected molecular mass of 56.6 kDa and a pI value of 6.22. The corresponding sequence of γECS of bean nodules was 508 amino acids long, with a predicted mass of 57.6 kDa and a pI value of 6.12. The deduced amino acid sequences of γECS from pea and bean nodules shared 85% to 88% identity with those of tomato, *B. juncea*, and Arabidopsis, and identities reached 88% to 93% when only the sequences of the three legumes were compared (Fig. 10). The sequences included the putative active site Cys residue, which is present in the γECS proteins of *Trypanosoma brucei, Caenorhabditis elegans,* yeast, and rat (Lueder and Phillips, 1996), in adittion to those of higher plants. The sequences of γECS from pea and bean nodules also included a putative transit peptide at the N terminus (Fig. 10), with a conserved cleavage-site motif (Ile-Val-Ala↓Ala) that is predicted to target γECS to the plastids (Gavel and von Heijne, 1990). The position of the cleavage site is consistent with the high variability of the putative signal peptide (51 residues for pea and 60 residues for bean) as opposed to the high identity of the γECS sequences from the Ile-Val-Ala-Ala motif to the C terminus (Fig. 10).

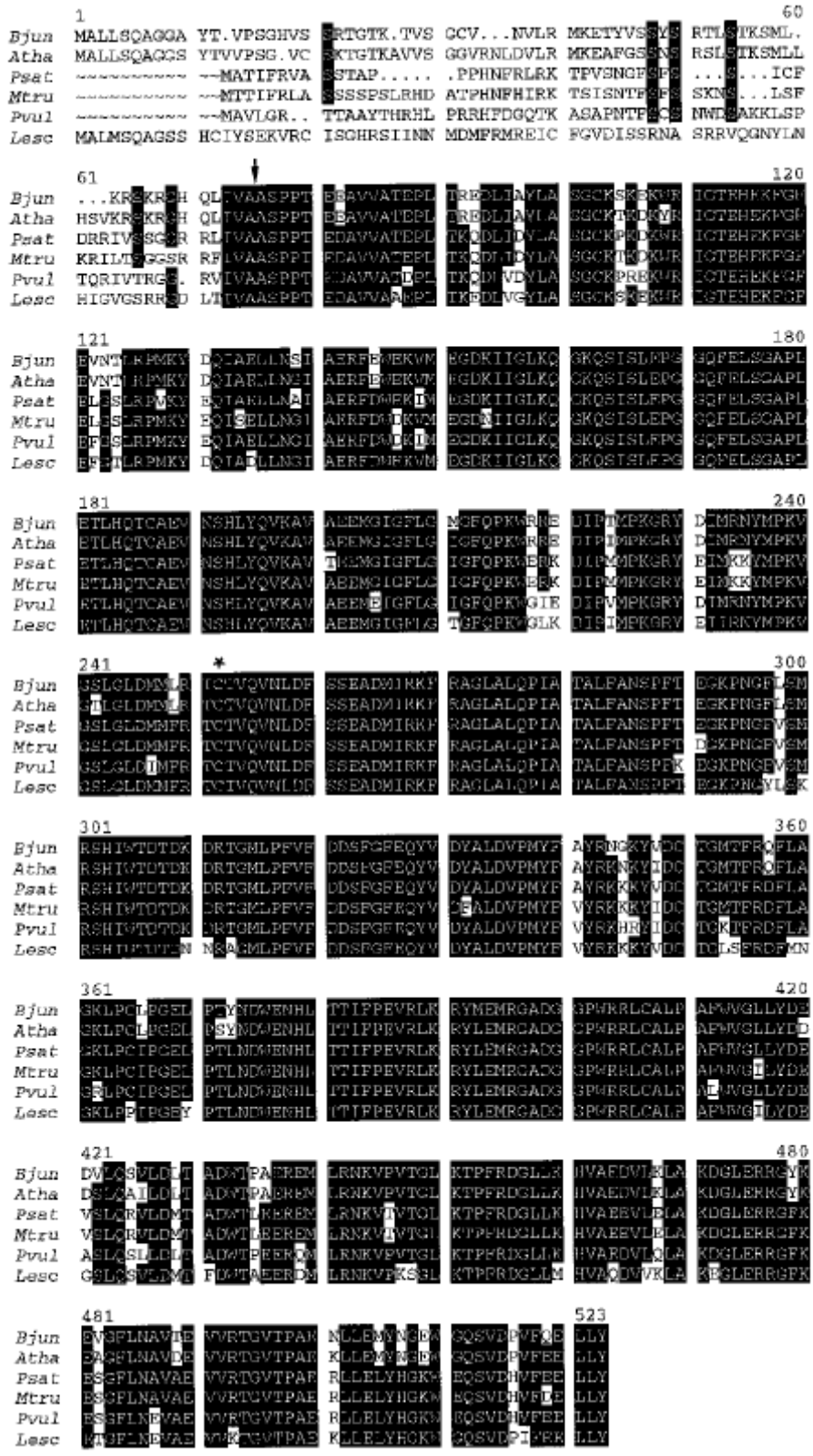


Figure 10. *Deduced amino acid sequences of γECS proteins from higher plants. Abbreviations and accession numbers are as follows: B. juncea (Bjun; accession no. Y10848), Arabidopsis (Atha; accession no. Y09944), P. sativum (Psat; accession no. AF128455), M. truncatula (Mtru; accession no. AF041340), P. vulgaris (Pvul; accession no. AF128454), and L. esculentum (Lesc; accession no. AF017983). Residues in white lettering on a black background are identical in at least four species. The putative cleavage site and active Cys residues are indicated by an arrow and asterisk, respectively.*

3.4. Discussion

Legume nodules contain higher concentrations of all non-protein thiols than the leaves and roots of the same plants, as well as high activities of all enzymes involved in GSH and hGSH synthesis, which indicates that the thiols are actively synthesized within the nodules. Furthermore, a comparison across legume species showed a close correlation between the major thiol tripeptide and the major synthetase activity present in the nodules, which strongly suggests that the relative abundance of GSH and hGSH is dictated by the distribution of the corresponding synthetases. This conclusion is supported by the strikingly different thiol tripeptide composition of alfalfa leaves (98% hGSH) and nodules (72% GSH). These results are consistent with the finding that GSHS activity is 4-fold higher than hGSHS activity in nodules but not detectable in leaves, and confirm that there are two synthetases, GSHS and hGSHS, in alfalfa nodules but a single enzyme, hGSHS, in alfalfa leaves. Indeed, two distinct thiol synthetases, identified on the basis of their different affinity for Gly and βAla, have been partially purified from pea (GSHS) and mungbean (hGSHS) leaves (Macnicol, 1987), which only contain GSH and hGSH, respectively (Klapheck, 1988; this work).

Very recently, Frendo et al. (1999) reported that the distribution of GSH and hGSH in *M. truncatula* plants is correlated with the expression of two genes with high homology to Arabidopsis *gshs,* reinforcing the view that two different enzymes, GSHS and hGSHS, are involved in GSH and hGSH synthesis. Although they do not provide details on how the level of hGSH, which is not commercially available, was estimated, their results indicate that the leaves of *M. truncatula* only contain GSH, whereas the nodules have both hGSH and GSH. Comparison of this finding with our thiol determination in alfalfa confirms our conclusion that the ability of legumes to synthesize hGSH has no taxonomic value.

An interesting finding in this study is that all nodules examined have substantial amounts of GSH, even in those legumes such as soybean, bean, and mungbean that contain hGSH exclusively in their leaves or roots. Some GSH of

nodules might have originated in the bacteroids, which may become partly broken during thiol extraction with methanesulfonic acid. If this is so, the total GSH content of nodules could be even greater after complete breakage of bacteroids and the hGSH/GSH ratio in the plant fraction of soybean, bean, and mungbean nodules would be also higher. In any case, the high concentrations of thiols in nodules relative to leaves and roots strongly suggest that GSH and hGSH play an important role in N_2 fixation. This hypothesis is reinforced by dissection experiments revealing that thiols are more abundant in the meristematic and infected zones than in the senescent zone of pea nodules, and more abundant in the infected zone than in the cortex of bean nodules.

The lower content of GSH in the senescent zone of pea nodules, however, cannot be explained only on the basis of reduced synthesis, since γECS activity was similar to that of the meristematic and infected zones, and the declines in Cys and GSHS activity were rather modest. The decline in GSH may have been due for the most part to enhanced degradation, because oxidative reactions, probably linked to the breakdown of heme and formation of green pigments from Lb (Roponen, 1970), are augmented in the senescent zone. The same process may also explain the decline in the average thiol concentration of whole nodules with advancing age or during stress-induced senescence, where nearly 90% of GSH (pea) and hGSH (bean) was lost.

The relatively high thiol concentrations and synthetase activities in the meristematic and early symbiotic zones of pea nodules may be physiologically relevant. Because active cell division is confined to the persistent meristems of indeterminate nodules, GSH may be involved in the control of cell proliferation in nodules, as proposed for the apical meristem of Arabidopsis roots (Sánchez-Fernández et al., 1997; May et al., 1998a). This does not preclude other possible functions of GSH in indeterminate nodules, such as the modulation of gene expression in the early symbiotic zone.

The consistently greater hGSHS activity in the cortex of bean nodules is puzzling but may provide a preliminary clue to elucidate the role of hGSHS and hGSH in determinate nodules. For example, it would be worth investigating whether the function of hGSHS is related to the vascular bundles, which are confined to the nodule cortex. Despite the remarkably high hGSHS activity, however, the cortex of bean nodules contained less hGSH than the infected zone. The control of GSH (and presumably hGSH) synthesis could be exerted by different mechanisms. These include the availability of the thiol precursors Cys and γEC and the amount of γECS enzyme (Rennenberg, 1997; May et al., 1998a). Because there is virtually no γEC in

the cortex and Cys content and γECS activity are only 21% and 41%, respectively, of those existing in the infected zone, the lower hGSH content in the cortex is probably due to a limitation of hGSH synthesis rather than to transport of the tripeptide into the infected zone. The relative availability of Gly and βAla for GSHS and hGSHS in the cortex and infected zone, as well as the possible contribution of bacteroids to GSH synthesis, may be also important in determining the different abundance of the thiol tripeptides in both nodule regions.

Another mechanism for the regulation of GSH synthesis is feedback inhibition of γECS by GSH (Rennenberg, 1997; May et al., 1998a; Noctor and Foyer, 1998). The estimated concentrations of GSH plus hGSH in the infected zone of pea and bean nodules are, respectively, 2.6 mM and 0.7 mM, which would be sufficient to inhibit γECS in vitro (Hell and Bergmann, 1990). However, our data do not support the idea that this inhibition occurs in vivo, since the extractable γECS activities are in fact greater in the infected zone of both nodules. The lack of apparent inhibition of γECS by GSH in vivo has been described in other plant systems, but a conclusive explanation has not yet been offered (Rennenberg, 1997; May et al., 1998a). Thus, the inhibition of γECS might be overcome by high concentrations of Glu, which competes with GSH (Hell and Bergmann, 1990; Rennenberg, 1997). Another possibility is that the relative concentrations of GSH and γECS differ in the cytosol and organelles of nodules, as occurs in pea leaves, where 72% of γECS activity (Hell and Bergmann, 1990) but only 10% of total GSH (Bielawski and Joy, 1986) are located in the chloroplasts.

The reaction catalyzed by γECS is generally assumed to be the rate-limiting step in GSH synthesis (May et al., 1998a; Noctor and Foyer, 1998), and indeed the γECS activities extractable from nodules were in most cases lower than those of the predominant GSHS or hGSHS enzymes. Like γECS from other sources, the nodule enzyme was irreversibly inhibited by buthionine sulfoximine, indicating that the reaction proceeds through the formation of an enzyme-bound γ-glutamyl-phosphate intermediate (Huang et al., 1988; Hell and Bergmann, 1990). Nodule γECS was not inhibited by low (0.48 mM) or high (5 mM) DTE concentrations. The same result was observed in leaves of Arabidopsis and maize (May and Leaver, 1994), while inhibition by DTE was found in tobacco suspension cells and in spinach and pea leaves (Hell and Bergmann, 1990). The lack of inhibition by this thiol reagent suggests that nodule γECS is a monomeric enzyme, unlike γECS from tobacco cells, which dissociates into two equal and inactive subunits in the presence of 5 mM DTE (Hell and Bergmann, 1990).

The derived amino acid sequences of γECS from pea and bean nodules were highly homologous to those of other higher plants and contained the purported active site Cys residue shared by the γECS proteins of species as evolutionary distant as the protozoans, yeasts, plants, and mammals (May and Leaver, 1994; Lueder and Phillips, 1996; May et al., 1998a). The sequences also included a putative plastid signal peptide of 50 to 60 amino acids, as suggested by the matching to a conserved cleavage-site motif, by the relatively high content of Ser plus Thr and Ala (but also Arg) residues, and the almost complete absence of acidic residues (von Heijne et al., 1989; Gavel and von Heijne, 1990). The variability of the putative signal peptides is somewhat surprising, as it is the finding that the signal peptides of legumes are significantly shorter than those of the other higher plants. The plastid location of legume nodule γECS will need to be verified by subcellular fractionation, N-terminal sequencing of the mature protein, or import in vitro. This is important because predictive algorithms for the subcellular localization of proteins such as PSORT (Nakai and Kanehisa, 1992) suggest compatibility also with a mitochondrial and peroxisomal targeting of γECS.

The instability and low abundance of γECS in plant tissues have hampered the complete purification of the enzyme and the study of mechanisms controlling γECS expression and activity in plants. Because thiol metabolites are actively synthesized and required for nodule functioning, experiments are under way to obtain recombinant γECS proteins and use them to investigate the regulatory mechanisms of thiol synthesis in legume nodules.

4. *Glutathione and homoglutathione synthetases of legume nodules: cloning, expression, and subcellular localization*

4.1. Introduction

The thiol tripeptide GSH is very abundant in plants, where it performs a multiplicity of important functions ranging from scavenging of reactive oxygen species to heavy metal detoxification (Hausladen and Alscher, 1993; Rennenberg, 1997; May et al., 1998a). The synthesis of GSH involves two reactions catalyzed by γECS and GSHS which are strictly dependent on ATP and Mg^{2+} (Fig. 11). In the leaves, the synthesis of GSH is thought to take place in both the chloroplasts and cytosol (Hausladen and Alscher, 1993; Rennenberg, 1997; Noctor and Foyer, 1998).

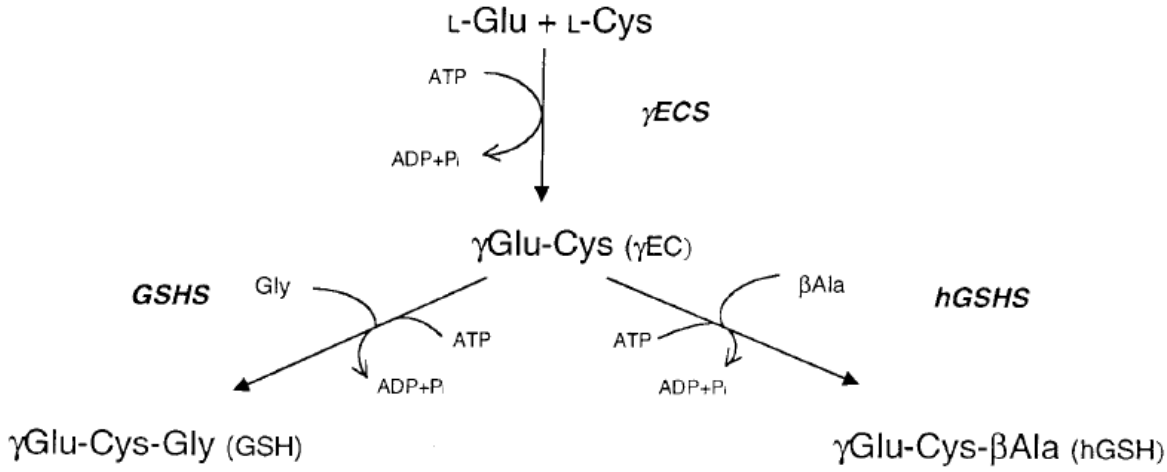


Figure 11. *Pathway for GSH and hGSH synthesis in legumes. The two ATP-dependent reactions leading to GSH synthesis are, respectively, the condensation of Glu and Cys to form γEC (catalyzed by γECS) and the addition of Gly to the C-terminus of γEC (catalyzed by GSHS). For hGSH synthesis, βAla replaces Gly in the second reaction (catalyzed by hGSHS).*

Legumes may contain another thiol tripeptide, hGSH, partially or fully replacing GSH (Fig. 11). The synthesis of hGSH from γEC and βAla is catalyzed by a specific hGSHS with high affinity for βAla and low affinity for Gly (Macnicol, 1987; Klapheck et al., 1988). Based on analyses of thiol metabolites and thiol synthetase activities in different organs and nodule tissues of eight legumes of agronomic

relevance, we proposed the hypothesis that GSH plays a critical role in N_2 fixation (Matamoros et al., 1999b). As an essential step to elucidate this role, we have initiated the molecular study of γECS, GSHS, and hGSHS of legume nodules. Using a strategy combining PCR-screening of nodule cDNA libraries, 5'-RACE, and reverse-transcription PCR (RT-PCR) of nodule and leaf RNA, we have obtained the complete cDNA sequences encoding the three enzymes from the nodule host cells. Results showed that the synthesis of GSH and hGSH in nodules involve the participation of several cell compartments. Subcellular fractionation studies also showed that bacteroids contain high thiol concentrations and the highest specific activities of γECS and GSHS for any nodule fraction, providing further evidence that GSH is critical for nodule functioning.

4.2. Materials and methods

4.2.1. Plant growth

Nodulated pea (*Pisum sativum* L. cv Lincoln x *Rhizobium leguminosarum* biovar *viciae* NLV8), bean (*Phaseolus vulgaris* L. cv Contender x *Rhizobium leguminosarum* biovar *phaseoli* 3622), soybean (*Glycine max* Merr. cv Williams x *Bradyrhizobium japonicum* USDA110), and cowpea (*Vigna unguiculata* Walp. cv California #5 x *Bradyrhizobium* sp.*(Vigna)* 32H1) plants were grown under controlled environment conditions as described (Gogorcena et al., 1997). All legumes were at the vigorous vegetative growth stage (30 to 35 d) when leaves and nodules were harvested. Leaves and nodules to be used for RT-PCR experiments were collected with gloves and immediately frozen in liquid nitrogen. All plant material was stored at -80°C except nodules to be used for dissection and subcellular fractionation studies, which were processed immediately after harvest.

4.2.2. Thiol synthetase assays

Thiol compounds were derivatized with MBB and quantified by HPLC with fluorometric detection (Fahey and Newton, 1987) with the modifications described in detail elsewhere (Matamoros et al., 1999b). Thiol synthetase activities in nodule extracts and organelles were determined using the same HPLC method based on the γEC synthesized from Cys and Glu (γECS), GSH synthesized from γEC and Gly (GSHS), and hGSH synthesized from γEC and βAla (hGSHS). The optimized extraction and assay media for the enzymes were identical to those previously reported (Matamoros et al., 1999b), with the only exception that the DTE concentration for the assay of γECS activity was increased from 0.5 mM to 5 mM.

Enzyme activities in all nodule fractions and organelles were expressed on a protein basis. Protein was quantified by the dye-binding microassay (Bio-Rad Laboratories, Hercules, CA), using BSA as a standard.

4.2.3. Isolation of cDNA clones encoding thiol synthetase cDNAs

The cDNA clones encoding γECS were isolated by PCR screening of nodule libraries using oligonucleotide primers designed to conserved sequences (GenBank accession numbers in parenthesis) of Arabidopsis (Y09944), tomato (AF017983), and *B. juncea* (Y10848). Pea and bean λZAP cDNA libraries were provided by Dr. Carroll Vance (U.S. Department of Agriculture-University of Minnesota, St. Paul), and the soybean λgt11 cDNA library by Dr. Robert Klucas (University of Nebraska, Lincoln).

The same libraries were PCR-screened to isolate cDNA clones encoding GSHS and hGSHS of legume nodules. Degenerate primers were designed based on the complete GSHS cDNA sequences of Arabidopsis (U22359, AJ243813), tomato (AF017984), and *B. juncea* (Y10984), and on the partial GSHS cDNA sequences of *M. truncatula* (AF075699, AF075700). Primers used to isolate internal cDNA sequences were: sense: 5'-CG[A/C]AACATGTA[C/T]GA[C/T]CA[A/G]CATT-3'; antisense: 5'-CCTTCTCT[C/T]TG[A/G]GG[C/T]TTCAT-3'. The 5'- and 3'-ends of all nodule clones, except those of pea nodule *GSHS2*, were amplified using the above primers in combination with T3 and T7 primers. The PCR mixture contained 0.5 μM of primers, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.05% (v/v) W-1 detergent, and 1.5 units of *Taq* polymerase (Life Technologies, Paisley, UK), in a final volume of 25 μL of PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl). PCR conditions were exactly as previously described (Matamoros et al., 1999b).

Primers used for PCR amplification of the 5' and 3' ends of pea nodule *GSHS2* cDNA were: sense: 5'-GCAGTCGCAATCGTTTACTTCC-3', antisense: 5'-CCCACCTTCATCAAATAATGATGG-3'. The PCR mixture contained 0.2 μM of both primers, 0.24 mM dNTPs, 1.5 mM $MgCl_2$, 0.05% W-1 detergent, and 1.25 units of *Taq* polymerase, in a final volume of 25 μL of PCR buffer. The PCR cycling protocol consisted of an initial denaturation step at 95°C for 3 min, 40 cycles (95°C for 45 s, 62°C for 45 s, 72°C for 90 s), and a final elongation step at 72°C for 10 min.

4.2.4. RACE-PCR and RT-PCR

For 5'-RACE the manufacturer's instructions (Life Technologies) were followed using the primer 5'-CCTTCTCT[C/T]TG[A/G]GG[C/T]TTCAT-3' to generate specific cDNA. For the subsequent PCR of pea nodule *GSHS1*, pea nodule *GSHS2*, and bean *GSHS2*, the antisense primers 5'-CGGAAGAAGAACAAG AATCGTCG-3', 5'-TGGTGTATAGCCAGCTCGGAAG-3', and 5'-CCAAACTCACA CGATCAACAAGC-3' were used, respectively.

Total RNA was extracted from nodules using the hot phenol method followed by LiCl precipitation (de Vries et al., 1982). For the RT-PCR analysis of leaves and nodules, total RNA (5 µg) was treated with 2 units of DNase I at 37°C for 10 min to remove traces of contaminating DNA. After addition of 2.5 mM EDTA, samples were incubated at 65°C for 15 min to inactivate DNase. For reverse transcription, RNA samples were annealed to the primer 5´-CTCGAGGATCCGCGGCCGC-$(T)_{20}$-3´at 70°C for 10 min, and then the cDNAs were synthesized using 200 units of reverse transcriptase (Superscript, Life Technologies) in a buffer containing 10 mM dithiothritol and 1.25 mM dNTPs. The reaction proceeded at 42°C for 55 min and was stopped at 70°C for 15 min. The remaining RNA present in the samples was removed by incubation with 1 unit of RNase H at 37°C for 20 min. The reaction mix was diluted to 120 µL, and 5 µL was used as template for PCR amplification.

For the PCRs, gene specific primers were designed based on the UTR sequences. Oligonucleotides used were as follows. For pea *GSHS1*, sense: 5'-CCCCTTTCTTCTCCAAACACATTC-3', antisense: 5'-CGGAAGAAGAACAAG AATCGTCG-3'. For pea *GSHS2*, sense: 5'-GTTGTTGATTGATGGCTTGCATG-3', antisense: 5'- GCGCCAAAATCCATTGTGAAC-3'. For bean *GSHS2*, sense: 5'-GAAAGTGGCTATATGGTGCG-3', antisense: 5'-GACACCATTCAGTAGGAA AAGC-3'. The reaction mixture contained 5 µL of first-strand cDNA, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 µM of primers, and 1.25 units of *Taq* polymerase (Perkin-Elmer) in a total volume of 25 µL. The PCR cycling conditions comprised an initial denaturation step at 94°C for 2 min, 30 to 35 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 45 s), and a final elongation step at 72°C for 10 min. As an internal control, PCR was performed simultaneously using ubiquitin primers (Horvath et al., 1993). In all cases, preliminary runs were used to verify that the number of amplification cycles was well below that required for signal saturation.

4.2.5. Cloning and sequencing

The cDNA bands of the expected sizes were gel purified (Concert, Life Technologies, or QIAquick gel extraction kit, Qiagen, Santa Clarita, CA) and subcloned into pCRII or pCR2.1. All sequencing was conducted on both strands of cDNA from at least two clones with an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA) using AmpliTaq DNA polymerase, FS dye-terminator cycle sequencing chemistry. Homology searches were done with the BLAST algorithm (Altschul et al., 1997). Sequence alignments and homology analyses were performed using the PileUp and Gap programs, respectively, of the Genetics Computer Group (10.0) package (Madison, WI). Phylogenetic analysis was performed with the CLUSTAL W (1.75) suite of programs (Thompson et al., 1994). Signal peptide analyses and predictions of subcellular localization were performed using the programs MitoProtII (Claros, 1995), PSORT (Nakai and Kanehisa, 1992), and ChloroP and TransitP (Center for Biological Sequence Analysis, Department of Biotechnology, Technical University of Denmark, Denmark).

4.2.6. Organelle purification for assay of thiol synthetases

Nodule host-cell organelles and bacteroids were purified from nodules at 0 to 4°C using Percoll gradients. For the purification of the mitochondria and peroxisomes, nodules (10 g) were gently ground in a mortar with 30 mL of a medium containing 0.3 M mannitol, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 20 mM $MgCl_2$, and 2% (w/v) polyvinylpolypyrrolidone. The homogenate was filtered through four layers of cheesecloth (moistened with extraction medium). An aliquot (1 mL) of the filtrate was sonicated in an ice-bath (4 x 30 s with 30 s breaks; Branson sonifier) and centrifuged at 13,000*g* for 5 min. The cleared supernatant ("crude extract") was saved for enzyme analysis. The rest of the homogenate was centrifuged twice at 4,000*g* for 5 min and then at 12,000*g* for 15 min. An aliquot of the supernatant ("cytosol") was also saved for subsequent enzyme analysis. The pellet was washed with 25 mL of washing medium (extraction medium omitting polyvinylpolypyrrolidone) and resuspended in 1.8 mL of washing medium. The whole volume was loaded on a first Percoll gradient essentially as described by Sandalio et al. (1987). Peroxisomes, which banded between the 35% and 50% (v/v) Percoll layers, were freed from Percoll with two washes with washing medium. Mitochondria, which banded between the 15% and 35% (v/v) Percoll layers (Fig. 12), were freed from Percoll as for peroxisomes and loaded on a second Percoll gradient following the method of Struglics et al. (1993). Mitochondria and peroxisomes were

broken by resuspension in 0.5 mL of hypotonic medium (50 mM Tris-HCl [pH 8.0], 0.2 mM EDTA, and 20 mM $MgCl_2$) and overnight incubation at 0°C. Broken organelles were then centrifuged and immediately used for enzyme analyses. Freezing and thawing of organelles did not significantly affect the yield and activity of enzymes.

Figure 12. *Purification of bean nodule mitochondria. The photograph shows the band of highly purified intact mitochondria after the first Percoll gradient.*

For plastid purification, nodules (10 g) were carefully ground in a mortar with 30 mL of a medium containing 0.3 M sucrose, 30 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 20 mM $MgCl_2$. The homogenate was filtered through one layer of cheesecloth and centrifuged at 3,000*g* for 5 min. The pellet was resuspended in 25 mL of extraction medium. After a new centrifugation at 200*g* for 5 min, the pellet was discarded and the supernantant was centrifuged at 3,000*g* for 5 min. The plastid-enriched pellet was resuspended in 2 mL of extraction medium and the plastids were purified by using sequentially two 35% (v/v) Percoll gradients as described by Atkins et al. (1997). Plastids were broken as indicated for mitochondria and peroxisomes.

For bacteroid purification, nodules (1 g) were carefully ground in a mortar with 1 mL of a medium containing 50 mM KH_2PO_4 and 150 mM NaCl (pH 8.0). The residue was further ground with 1 mL of the same medium and the pooled extract was filtered through four layers of cheesecloth. The extract (1.5 mL) was loaded on 70% (v/v) Percoll made in extraction buffer, and bacteroids were purified as described by Reibach et al. (1981). Bacteroids were broken by sonication (4 x 30 s with 30 s breaks) in an ice-bath. For the thiol analysis of bacteroids, the same extraction buffer was used except that the pH was adjusted to 6.5. The purified bacteroids were broken by resuspension in 200 mM methanesulfonic acid (containing 0.5 mM diethylenetriaminepentaacetic acid) and by subsequent sonication.

4.2.7. Organelle purification for assay of marker proteins

Similar procedures, but at pH 7.2, were used to monitor the purification process of organelles with the assistance of marker proteins: β-hydroxybutyrate dehydrogenase and alanine dehydrogenase (bacteroids; Reibach et al., 1981), Cyt *c* oxidase (mitochondria; Schnarrenberger et al., 1971), uricase and catalase (peroxisomes; Hanks et al., 1981), NADH-glutamate synthase (plastids; Atkins et al., 1997), and Lb (cytosol; La Rue and Child, 1979). Nodule crude extracts, cytosol, mitochondria, and peroxisomes were purified using an extraction medium comprising 0.35 M mannitol, 30 mM MOPS (pH 7.2), 2 mM EDTA, 10 mM KH_2PO_4, and 2% (w/v) polyvinylpolypyrrolidone. The purified organelles were washed with the same medium omitting polyvinylpolypyrrolidone. Plastids were purified using an extraction and washing medium containing 0.3 M sucrose, 30 mM MOPS (pH 7.2), 1 mM EDTA, and 20 mM $MgCl_2$. Bacteroids were purified using an extraction and washing medium containing 50 mM KH_2PO_4 and 150 mM NaCl (pH 7.2).

To assay β-hydroxybutyrate dehydrogenase and alanine dehydrogenase, nodule crude extracts and purified fractions were sonicated 4 x 30 s (with 30 s breaks) in an ice-bath. To assay Cyt *c* oxidase, uricase, catalase, and Lb, nodule extracts and fractions were made to 0.05% (v/v) Triton X-100. To assay NADH-glutamate synthase, nodule extracts and fractions were made (immediately after isolation) to 94 mM MES (final pH 6.5), 0.05% (v/v) Triton X-100, and 270 mM β-mercaptoethanol. This enzyme was found to be labile at pH 8.0 and in the absence of β-mercaptoethanol, as previously reported by others (Groat and Vance, 1981).

4.3. Results

4.3.1. Isolation of cDNAs and sequence analyses of thiol synthetases

We have previously reported the isolation of cDNAs encoding γECS of pea and bean nodules (Matamoros et al., 1999b). To complete the molecular study of nodule thiol synthetases, the first part of this work was devoted to isolate cDNA clones encoding the enzymes GSHS and hGSHS, which catalyze the second step of GSH and hGSH synthesis in legumes (Fig. 11). Screening of a pea nodule library by PCR with primers based on conserved sequences of GSHS from other higher plants produced a number of positive clones. The cDNA inserts were sequenced and shown to correspond to two different genes. The complete sequences of the cDNAs, designated *GSHS1* and *GSHS2,* were obtained and 5'-RACE analysis used to confirm the starting ATG codons. Pea *GSHS1* and *GSHS2* shared 74% identity and both were approximately 65% identical with the homologous complete cDNAs of Arabidopsis, *B. juncea,* and tomato. The pea sequences were also 74 to 88% identical with two partial sequences obtained from a *M. truncatula* cDNA library made from 4-d old nodules (Frendo et al., 1999) and with a full-length sequence of soybean recently deposited in the databases (accession no. AJ272035). Pea *GSHS1* has an ORF of 1662 bp, which encodes a 552 amino acid polypeptide, with a calculated molecular mass of 61.5 kDa and pI of 6.50. Pea *GSHS2* has an ORF of 1491 bp, which encodes a 495 amino acid polypeptide, with a molecular mass of 55.9 kDa and pI of 5.54.

The same primers were used to screen a bean nodule library, but in this case only cDNA clones corresponding to a single gene could be isolated. All bean nodule cDNA clones examined were truncated at the 5' end. The sequence was completed by 5'-RACE, which provided 19 bp extra in the ORF (including the starting ATG) and 9 bp in the 5'-UTR. The complete bean sequence showed approximately 63% identity with the GSHS cDNAs of Arabidopsis, *B. juncea,* and tomato, 73% identity with pea *GSHS1* and *GSHS2*, and 72 to 80% identity with the sequences of *M. truncatula* and soybean. The bean sequence contains an ORF of 1635 nucleotides, encoding a 543 amino acid polypeptide, with a calculated molecular mass of 60.2 kDa and pI of 5.64 (Table 9). At the protein level (Fig. 13), it shows higher homology with pea GSHS2 (73% identity) than with pea GSHS1 (66% identity), and the bean nodule cDNA was therefore designated *GSHS2.* Similarly, the soybean enzyme was designated as GSHS2 because of its higher homology at the protein level with pea GSHS2 (76.2% identity) and bean GSHS2 (91.1% identity) than with pea GSHS1 (71.3%).

Table 9. *Predicted properties of thiol synthetases from pea and bean nodules*

Enzyme	Length[a]	Signal[b]	M_r[c]	pI[d]	Cleavage site	Subcellular localization
Pea γECS	499	51	56.6	6.22	RLIVA↓ASPP	Plastids
Bean γECS	508	60	57.6	6.12	RVIVA↓ASPP	Plastids
Pea GSHS1	552	20	61.5	6.50	FFSKH↓IPST	Mitochondria
Pea GSHS2	495	---	55.9	5.54	-----	Cytosol
Bean GSHS2	543	59?	60.2	5.64	NSAPL↓AEPD?	Plastids and/or cytosol

[a] Number of amino acid residues of precursor protein. [b] Number of amino acid residues of putative signal peptide. [c] Molecular mass of precursor protein. [d] Isoelectric point of precursor protein.

The ORFs of pea *GSHS1* and *GSHS2* and of bean *GSHS2* have two stop codons and identical polyadenylation sequences (AATAAA) at the 3' ends. All three cDNAs isolated have also abundant 3'-UTR material preceding the poly(A^+) tail, but short sequences in the 5'-UTR (see accession nos. in legend to Fig. 13).

4.3.2. Predicted properties and phylogenetic analysis of thiol synthetases

The predicted properties of thiol synthetases from nodules are indicated in Table 9. The *M. truncatula* enzymes were not included because the corresponding cDNAs lack the 5'-regions and therefore no predictions can be made with respect to their subcellular localization. The γECS proteins contained a putative cleavage site motif, Ile-X-Ala↓Ala , for plastid targeting. We have now confirmed these findings with the isolation of a soybean γECS cDNA bearing the complete 5' end (accession no. AF128453). The deduced amino acid sequence of soybean γECS also contained, at the N-terminus, a cleavage site motif (Ile-Val-Ala↓Ala) and a plastid transit peptide (56 amino acids). In contrast, no such motif was found for any of the GSHS proteins from nodules (Fig. 13). Prediction programs indicated that pea GSHS2 has no signal peptide and is localized in the cytosol, and that pea GSHS1 has a signal peptide for mitochondrial, rather than plastidic, targeting. However, the putative subcellular localization of bean GSHS2 is more ambiguous. The PSORT program gave rather similar probabilities for a localization in the plastids and cytosol, whereas ChloroP indicated a plastidic localization with a putative cleavage site between residues 59 and 60. The sequence alignment shown in Figure 13 shows that the length of bean GSHS2 is similar to those of Arabidopsis GSHSp, *B. juncea* GSHS, and *L. esculentum*

GSHS, all of which are predicted to be localized in organelles. As expected, the four sequences are in turn considerably longer than those of the cytosolic enzymes, pea GSHS2 and Arabidopsis GSHSc. All these comparisons strongly suggest that bean GSHS2 bears a signal peptide.

The programs PSORT, ChloroP, and MitoProtII were also used, as a control, to predict the subcellular localization of the other plant GSHS proteins shown in Figure 13. The programs correctly localized Arabidopsis GSHSp and *L. esculentum* GSHS in the chloroplasts; however, they predicted that *B. juncea* GSHS, assumed to be a mitochondrial enzyme (Schäfer et al., 1998), is targeted to the plastids and that soybean GSHS2, assumed to be a plastidic enzyme (accession no. AJ272035), is cytosolic. The latter case is also evidenced by an almost identical length of soybean GSHS2 and pea GSHS2 (Fig. 13).

In addition, the criteria of von Heijne et al. (1989), based on the Ser/Arg ratio to discriminate between plastidic and mitochondrial transit peptides, identified also Arabidopsis GSHSp and *B. juncea* GSHS as plastidic enzymes and pea GSHS1 as a mitochondrial enzyme. Figure 13 also shows that there is considerable homology among the complete GSHS sequences of all higher plants examined from residue 92 onwards (numbering is based on the pea GSHS1 sequence) and little homology before residue 92, which includes the purported signal peptides. There was also relatively low homology in short streches interspersed in the proteins, particularly between residues 131 and 142, 233 and 266, 302 and 331, and 483 and 500.

The deduced GSHS sequences of plants, including a complete sequence of soybean GSHS2 and the two partial sequences of *M. truncatula*, were used to construct an unrooted phylogenetic tree (Fig. 14). Sequences were aligned using PileUp and analyzed using CLUSTAL W. The tree reveals that legume GSHS proteins cluster together with respect to the non-legume proteins, which in turn cluster in two groups. However, the most interesting results for the purposes of this paper are that, within the legumes, the GSHS1 and GSHS2 proteins cluster separately, and that, within GSHS2 proteins, those from legumes with determinate nodules (Phaseoleae) group separately from those with indeterminate nodulation (Vicieae, Trifolieae).

4.3.3. Sequence assignment and expression of thiol synthetases

Previous work showed that legumes contain exclusively either GSH or hGSH in their leaves and that the distribution of thiols in nodules is determined by the respective synthetases (Matamoros et al., 1999a,b). We then reasoned that the leaves

also express a single thiol synthetase and this was demonstrated by measuring enzyme activities in pea, bean, and cowpea leaves (Table 10). Pea leaves express only GSHS and bean leaves only hGSHS. This finding was very useful to assign the cDNA sequences of pea and bean nodules to either the GSHS or hGSHS groups of enzymes. Cowpea was introduced at this stage of the study because we needed, for localization studies, an additional legume species producing exclusively GSH and amenable for subcellular fractionation of nodules.

Table 10. *Thiol tripeptide synthetase activities in legume leaves and nodules. Values are means ± SE of three to six samples obtained from two series of independently-grown plants. L, leaves; N, nodules. Enzyme activities are expressed in nanomoles per minute per gram fresh weight.*

Enzyme	Pea		Bean		Cowpea	
	L	N[a]	L	N[a]	L	N[a]
GSHS	5.0 ± 0.2	11.4 ± 1.2	0	0.7 ± 0.2	10.5 ± 3.5	15.8 ± 1.8
hGSHS	0	6.9 ± 0.4	2.6 ± 0.1	7.0 ± 1.0	0	0

[a] Data taken from Table 6.

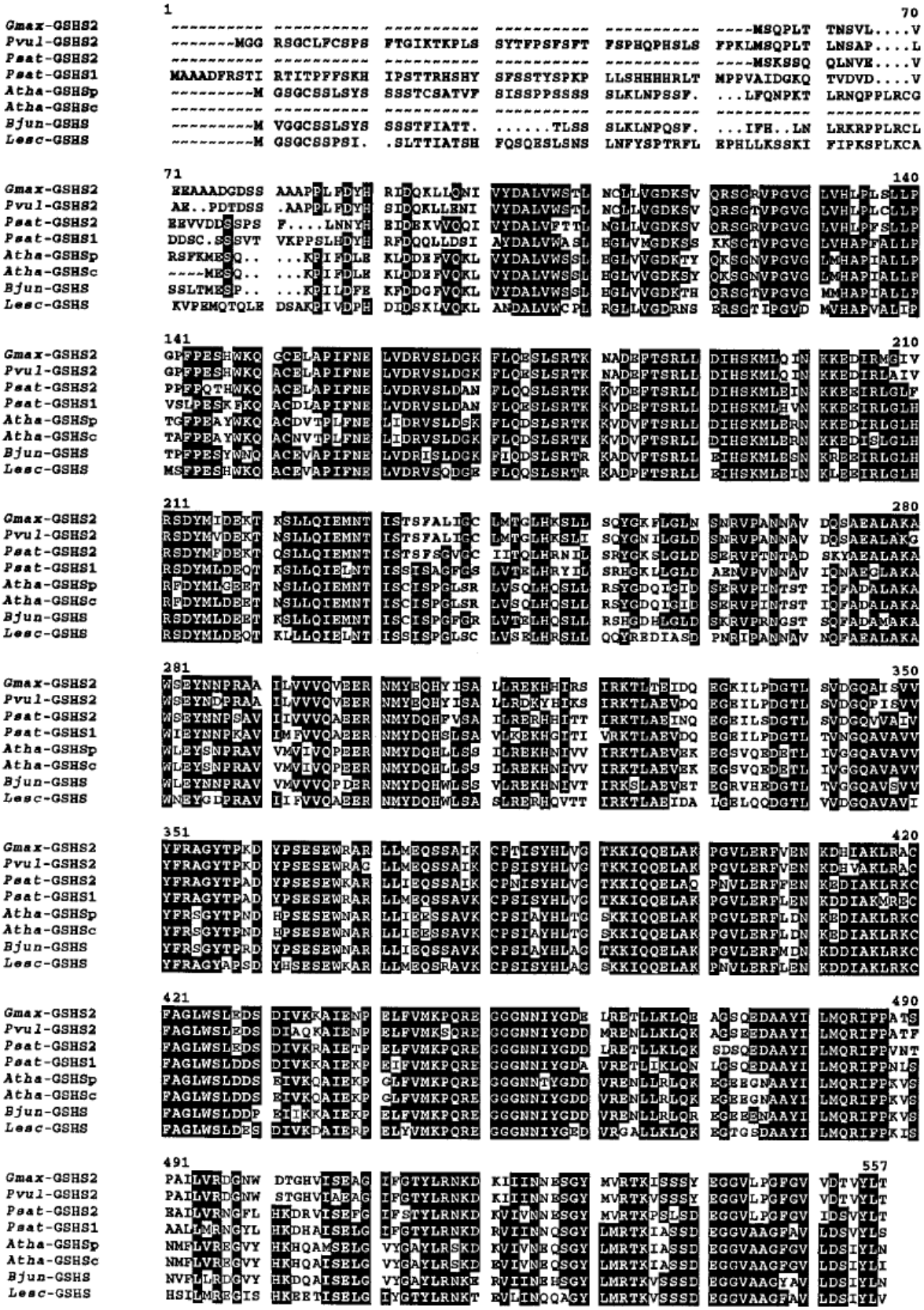

Gmax-GSHS2
Pvul-GSHS2
Psat-GSHS2
Psat-GSHS1
Atha-GSHSp
Atha-GSHSc
Bjun-GSHS
Lesc-GSHS

Figure 13. *Alignment of complete deduced amino acid sequences of GSHS and hGSHS from higher plants. Abbreviations and GenBank accession numbers are as follows. Glycine max GSHS (Gmax-GSHS2, AJ272035), Phaseolus vulgaris GSHS2 (Pvul-GSHS2, AF258320), Pisum sativum GSHS2 (Psat-GSHS2, AF258319), P. sativum GSHS1 (Psat-GSHS1, AF231137), Arabidopsis plastidic GSHS (Atha-GSHSp, AJ243813), Arabidopsis cytosolic GSHS (Atha-GSHSc, U22359), Brassica juncea GSHS (Bjun-GSHS, Y10984), and Lycopersicon esculentum GSHS (Lesc-GSHS, AF017984). Dots denote gaps to maximize alignment. Residues in white lettering on a black background are identical in at least five sequences.*

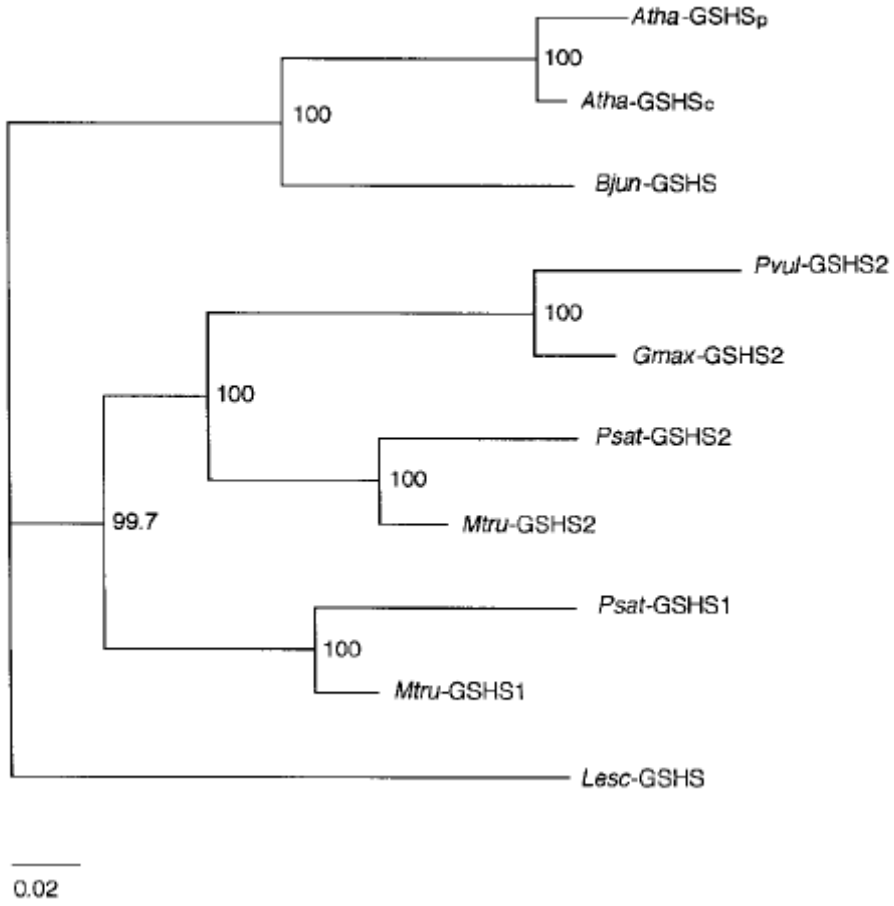


Figure 14. *Unrooted phylogenetic tree of GSHS proteins from higher plants. The tree was calculated using the neighbor-joining method of the CLUSTAL W suite of programs. The numbers correspond to percentages of 1000 'bootstraps'. The bar represents 0.02 subtitutions per site.*

RT-PCR analysis using gene-specific primers based on the 5'-UTR of pea *GSHS1* and 3'-UTR of pea *GSHS2* revealed that *GSHS1* is equally expressed in leaves and nodules, but *GSHS2* is expressed only in nodules (Fig. 15). Therefore, pea *GSHS1* encodes a GSHS, whereas the product of *GSHS2* can be tentatively identified as a hGSHS because pea plants express hGSHS in nodules but not in leaves (Table

10). The same analysis using primers based on the 3'-UTR sequence of bean *GSHS2* indicated that this gene is expressed at the same level in the leaves and nodules (Fig. 15). Consequently, bean *GSHS2* encodes a hGSHS. The assignments of pea GSHS1 as GSHS and of pea and bean GSHS2 as hGSHS were reinforced by the cluster analysis described above. Thus, *M. truncatula* GSHS2 (Frendo et al., 1999) and soybean GSHS2 (M. Skipsey, C.J. Andrews, J.K. Townson, I. Jepson, and R. Edwards, unpublished results) have been characterized as hGSHS enzymes. The two proteins cluster together with pea and bean GSHS2 and separately from GSHS1 (Fig. 14), indicating, together with our expression data and those of Frendo et al. (1999), that *GSHS2* encode hGSHS enzymes whereas *GSHS1* encode GSHS enzymes.

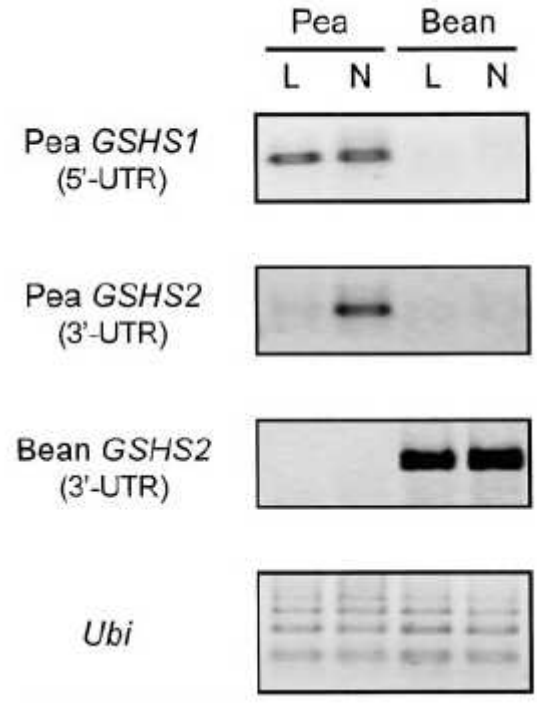


Figure 15. *Expression of GSHS in leaves and nodules of pea and bean plants. RT-PCR analysis was peformed using primers designed to 5'-UTRs or 3'-UTRs of pea (GSHS1 and GSHS2) and bean (GSHS2) cDNAs, as shown in the left. Ubiquitin (Ubi) was used as a control for uniform loading.*

The same gene-specific primers along with primers designed to the 3'-UTR of γECS cDNA were used to quantify expression of γECS, GSHS1, GSHS2 in pea nodules by RT-PCR. However, there were no major variations in the abundance of any of the three transcripts during natural (aging) or stress-induced nodule senescence (data not shown). This contrasts with the decline in the corresponding enzyme activities observed in aging pea nodules or the increase in γECS activity

observed in dark-stressed pea nodules (Matamoros et al., 1999b), and suggests that thiol synthetase activities may be regulated at the post-transcriptional level, as shown by May et al. (1998b) for γECS in Arabidopsis cell cultures.

4.3.4. Localization of thiol synthetases

The tentative localization of thiol synthetases in the various nodule compartments, based on the presence or absence of recognizable cleavage site motifs, required verification by purification of organelles on density gradients. Marker enzymes and Lb were used to assess cross-contamination among nodule organelles. Specific protocols had to be employed to purify plastids and bacteroids, whereas a single method served to purify mitochondria, peroxisomes, and cytosol. Mitochondria were <10% contaminated with peroxisomes and plastids, and showed no detectable contamination with the cytosol or bacteroids. Plastids showed <20% contamination with bacteroids, <10% with mitochondria and peroxisomes, and negligible contamination with cytosol. Chloroplasts, mitochondria, and cytosol were also purified from the leaves of the same plants to verify results. Similar protocols were followed for organelle purification, and chlorophyll and marker enzymes used to assess purity (Corpas et al., 1991; Jiménez et al., 1997). Chloroplasts were essentially free of contamination with cytosol or mitochondria. However, leaf mitochondria had still substantial contamination (20% to 30%) with thylakoids. Crude extracts of nodules and leaves, in which the enzymes were released from organelles by prolonged sonication, were also analyzed as parallel controls.

Bean nodule extracts showed γECS, GSHS, and hGSHS activities (Fig. 16A). However, when the extracts were not sonicated, only γECS and hGSHS activities could be detected, suggesting that GSHS activity originated in the bacteroids. Indeed, bean and cowpea bacteroids have very high GSHS activities (Fig. 16, A and B) and the small GSHS activity detected in the plastids of the two legumes (15% of the specific activity of bacteroids) was due to cross-contamination. Thus, when the plastid fractions were made up to 0.01% (v/v) Triton X-100 and centrifuged, the GSHS activity remained in the sediment (contaminating bacteroids) and not in the supernatant (broken plastids).

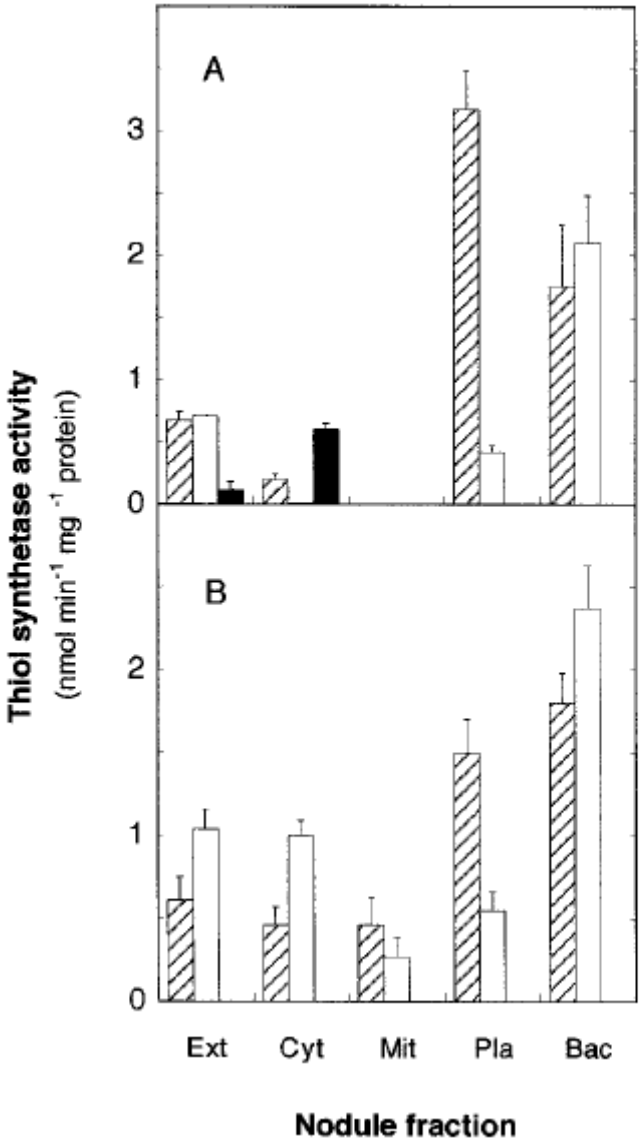


Figure 16. *Subcellular localization of (□) γECS, (□) GSHS, and (■) hGSHS in (A) bean and (B) cowpea nodules. Values are means ± SE of three or four independent experiments. Ext, Crude extract; Cyt, cytosol; Mit, mitochondria; Pla, plastids; Bac, bacteroids.*

Most γECS activity of bean nodules was localized in the plastids and bacteroids, with somewhat less activity being present in the cytosol; in contrast, the hGSHS activity was localized in the cytosol, with no measurable activity in the mitochondria, plastids, peroxisomes, or bacteroids (Fig. 16A). Assay of enzymes in nodules of soybean, another hGSH-producing legume (Matamoros et al., 1999b), confirmed that hGSHS activity was localized in the cytosol (data not shown). The same location was found for the hGSHS of bean leaves, with no measurable activity in chloroplasts or mitochondria (Fig. 17A). Furthermore, when large amounts of bean leaves were processed to partially purify hGSHS, we were unable to detect GSHS activity in the extracts and the hGSHS activity invariably remained in the

soluble fraction (cytosol) after sedimentation of organelles in isosmotic conditions. Consequently, the subcellular localization data demonstrate that hGSHS is the only thiol tripeptide synthetase present in bean leaves and nodule host cells, and that there is at least a hGSHS isoenzyme in the cytosol.

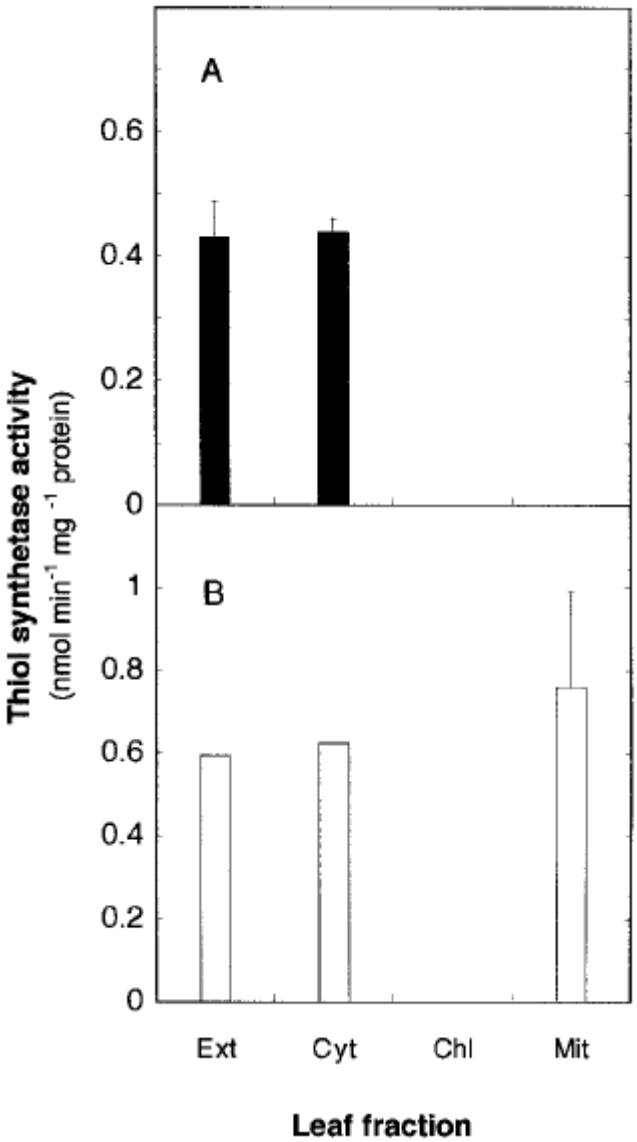


Figure 17. *Subcellular localization of (□) GSHS and (■) hGSHS in (A) bean and (B) cowpea leaves. Values are means ± SE of two or three independent experiments. Ext, Crude extract; Cyt, cytosol; Mit, mitochondria; Chl, chloroplasts.*

As could be anticipated for a GSH-producing legume (Matamoros et al., 1999b), crude extracts of cowpea nodules exhibited γECS and GSHS activities but not hGSHS activity (Fig. 16B). The majority of γECS activity was localized in the plastids and bacteroids, and the majority of GSHS activity in the cytosol and bacteroids. Unlike bean nodules, however, we found γECS and GSHS activities in the

mitochondria of cowpea nodules (Fig. 16B). Mitochondria preparations showed no detectable contamination with bacteroids and negligible contamination with plastids, and therefore we decided to purify leaf organelles to verify results. Subcellular fractionation of cowpea leaves revealed that mitochondria, but not chloroplasts, contain GSHS (Fig. 17B). This study also confirmed the presence of low levels of γECS in cowpea mitochondria (data not shown).

4.3.5. Thiols and thiol synthetases of bacteroids

Bacteroids purified on Percoll gradients were essentially free of contamination with host cell organelles or cytosol, as judged by the assay of marker proteins. Additional controls, consisting of bacteroids that had been washed up to four times or repurified on two sequential Percoll gradients prior to sonication, yielded identical results. Bacteroids showed high specific γECS and GSHS activities, but no hGSHS activity, regardless of the main thiol tripeptide synthetase present in the nodules (Fig. 16, A and B).

The high capacity of bacteroids for GSH synthesis and the lack of any previous information on their contribution as a source of thiols within the nodules, prompted us to measure the thiol content of bacteroids. Bean and cowpea nodule bacteroids contained 0.29 nmol Cys and approximately 10 nmol GSH per mg of protein (Table 11). As expected, cowpea bacteroids had no hGSH, but those bean contained 1.5 nmol hGSH per mg of protein. This small, but significant, hGSH concentration was not due to thiol adsorbed to bacteroid surface since it remained constant after repeated washings of bacteroids. Soybean bacteroids (strain USDA110) had significantly higher concentrations, 4.5 nmol hGSH per mg of protein. Because bacteroids do not express hGSHS (Fig. 16, A and B), we conclude that the hGSH found in the bacteroids was synthesized by the host plant and taken up through the symbiosome membrane.

Table 11. *Thiol contents of bacteroids from bean and cowpea nodules. Data are means ± SE of four to six samples of bacteroids isolated from at least two series of independently-grown plants. Thiol contents are expressed in nanomoles per milligram protein.*

Thiol[a]	Bean (strain 3622)	Cowpea (strain 32H1)
Cys	0.29 ± 0.05	0.29 ± 0.03
GSH	9.40 ± 0.68	12.70 ± 0.87
hGSH	1.53 ± 0.32	0

[a] The content of γEC was < 0.06 nanomoles per milligram protein in both legumes.

4.4. Discussion

Legumes are the only plants known to date containing hGSH in addition to or in place of GSH (Klapheck, 1988). The first enzyme committed to the synthesis of the thiol tripeptides, γECS, is ubiquitous in leaves and nodules, whereas expression of the second enzymes, GSHS and hGSHS, determines the relative abundance of GSH and hGSH in the different legume species and plant tissues (Matamoros et al., 1999a,b). To ascertain the specific roles of thiols in legume nodules and particularly in N_2 fixation, it is essential to characterize the genes and localize the three enzymes involved in their synthesis.

In this paper we demonstrate by subcellular fractionation that the γECS of nodule host cells is localized in the plastids (Fig. 16). Previous studies showed that the enzyme is present in the chloroplasts and cytosol of leaves (Hell and Bergmann, 1990) and in root plastids (Rüegsegger and Brunold, 1993). However, we found only very low γECS activity in the nodule cytosol and this is probably attributable to contamination with the enzyme of plastids, which are extremely fragile organelles (Atkins et al., 1997). There are some significant differences between the γECS from legume nodules and that from tobacco suspension cells. The nodule enzymes have a predicted molecular mass of approximately 51 kDa, significantly smaller than the 60 kDa found for the enzyme purified from tobacco (Hell and Bergmann, 1990). Likewise, the γECS activities of tobacco cells and of pea and spinach leaves, but not of Arabidopsis or maize (May and Leaver, 1994), were inhibited by reductants. In tobacco, the inhibition was due to dissociation of the protein into subunits (Hell and Bergmann, 1990). In contrast, we have observed that 5 mM DTE enhanced the extractable γECS activity of nodules from 1.2-fold to 3.5-fold (depending on species), which suggests that the nodule enzymes are active as monomers.

Knowledge on nonphotosynthetic plastids lags well behind that on chloroplasts partly due to difficulties encountered in their isolation (reviewed by Emes and Neuhaus, 1997). The same holds true for nodule plastids. As more biochemical and molecular information on nodule metabolism becomes available, the picture is emerging that plastids perform multiple functions essential for N_2 fixation. The best known functions of nodule plastids are related to their participation in ammonia assimilation (Temple et al., 1998) and purine synthesis (Atkins et al., 1997). We report here that another function, so far overlooked, is protection against reactive oxygen. Thus, the specific localization of γECS in plastids (Fig. 18), along with glutathione reductase (Tang and Webb, 1994), ferritin (Matamoros et al., 1999a) and Fe-superoxide dismutase (M.C. Rubio and M. Becana, unpublished results), emphasize that these organelles are a primary line of antioxidative defense in nodules. The three antioxidant proteins are responsive to stress and may be directly induced by reactive oxygen species (Lobréaux et al., 1995; May et al., 1998a; Matamoros et al., 1999a,b). In particular, γECS is the regulatory step of GSH synthesis and is post-transcriptionally activated in response to stressful conditions (May et al., 1998a,b). Therefore, plastids from nodules and probably from other non-green tissues have an important complement of antioxidant proteins which enable them to sense, and respond to, conditions generating oxidative stress in the plant.

Our results also indicate that the second step of GSH and hGSH synthesis in nodule host cells takes place predominantly in the cytosol (Fig. 18). This is only in partial agreement with earlier studies of subcellular localization in leaves. In pea and spinach leaves, between 47% and 69% of total GSHS activity is localized in the chloroplasts and the rest in the cytosol (Klapheck et al., 1987; Hell and Bergmann, 1990). We have found that the majority of GSHS in nodules and leaves of cowpea was localized in the cytosol. And, in fact, we have also isolated a cDNA clone from cowpea nodules that encodes a cytosolic GSHS (J.F. Moran and M. Becana, unpublished results). A novel observation is, however, that there is also GSHS in the mitochondria (but not in the plastids or chloroplasts) of cowpea leaves and nodules (Fig. 18), implying a further protective role of GSH against oxidants generated during respiration. In leaves, the specific activity of GSHS in mitochondria was, in fact, slightly higher than that in the crude extract and cytosol (Fig. 17B). The GSHS activity of nodule and leaf mitochondria is genuinely restricted to these organelles because they were not contaminated with bacteroids or nodule plastids, and because, although leaf mitochondria were contaminated with chloroplasts, these do not contain GSHS. The finding of GSHS activity in mitochondria is consistent with

the sequence analysis of pea nodule *GSHS1*, which encodes a protein bearing a putative mitochondrial transit peptide (Fig. 13).

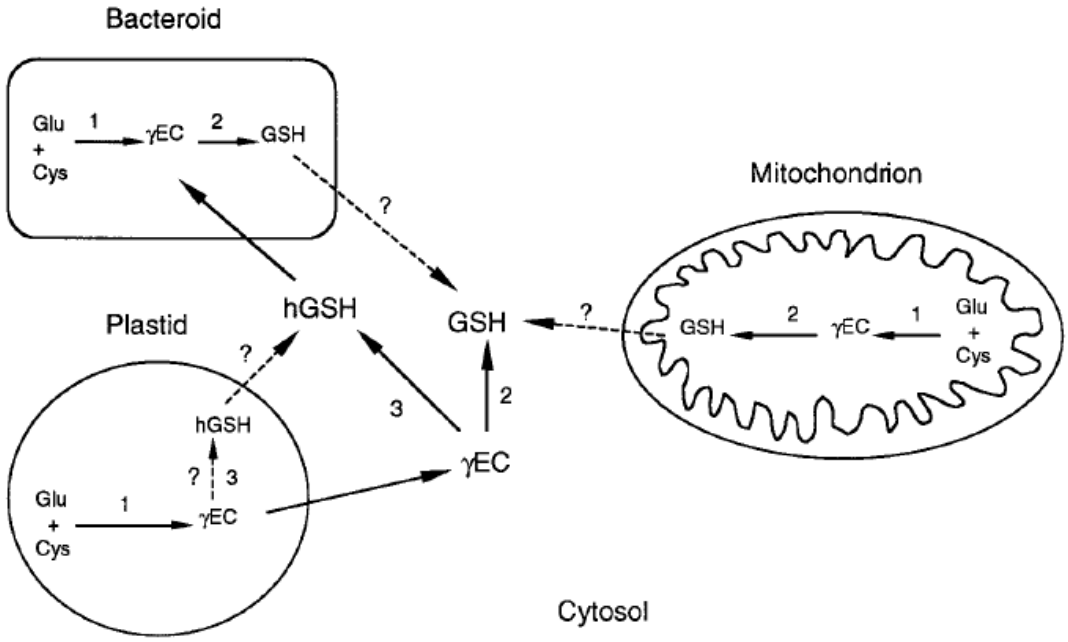


Figure 18. *Diagram depicting the subcellular localization of GSH and hGSH synthesis in nodules. Enzymes are (1) γECS, (2) GSHS, and (3) hGSHS. In GSH-producing legume nodules, reaction (1) occurs in the bacteroids, plastids, and mitochondria, and reaction (2) in the bacteroids, mitochondria, and cytosol. In hGSH-producing legume nodules, reaction (1) occurs in the bacteroids and plastids, reaction (2) in the bacteroids, and reaction (3) in the cytosol and possibly also in the plastids. Arrows in discontinuous lines indicate possible contributions of bacteroids and mitochondria to the cytosolic GSH pool and of the plastids to the cytosolic hGSH pool.*

We have found hGSHS activity only in the cytosol from nodules and leaves of bean (Figs. 16A and 17A) and soybean (data not shown). However, we cannot exclude the possibility that an additional hGSHS isozyme exists in the chloroplasts and nodule plastids in view of the cDNA sequence, bean *GSHS2*, that has been isolated in the course of this work. Although this sequence is also consistent with both a plastidic and cytosolic localization of the enzyme, it might be that the hGSHS activity in the chloroplasts and plastids is very low or unusually labile, escaping detection. This explanation would be more in agreement with, to our knowledge, the only previous report addressing hGSHS localization in plants. Klapheck et al. (1988) estimated that 17% of the hGSHS activity of *Phaseolus coccineus* leaves is in the chloroplasts, while the rest was assumed to be in the cytosol

We have also reported that the majority of hGSHS activity of bean nodules is in the cortex (Matamoros et al., 1999b) and have now expanded this observation to soybean nodules. These have distinctly different thiol synthetase activities (nmol $min^{-1}g^{-1}$ fresh weight) in the cortex (GSHS= 0, hGSHS= 9.9 ± 0.3) and in the infected zone (GSHS= 1.5 ± 0.8, hGSHS= 3.3 ± 1.4). Therefore, the predominant localization of hGSHS at the subcellular (cytosol) and tissue (nodule cortex) levels appears to be widespread in hGSHS-producing nodules.

Results of this work also reveal that bacteroids have very high γECS and GSHS activities and thiol concentrations (Fig. 16, Table 11), and therefore actively synthesize GSH (Fig. 18). These and previous findings (Matamoros et al., 1999b) suggest that bacteroids are a major source of GSH and would explain, at least in part, why this thiol is so abundant in the infected zone of both indeterminate and determinate nodules. In bacteroids, as in other procaryotes, GSH may have multiple functions. Very recently, one such function has been demonstrated. A *Rhizobium tropici* mutant strain containing 3% of the GSH present in the wild-type was more sensitive to osmotic and acid stress and was less competitive in coinoculation experiments, suggesting an important role of GSH in stress tolerance (Riccillo et al., 2000). Bacteroids cannot synthesize hGSH but this thiol, produced by the plant, can apparently cross the symbiosomal membrane and reach the bacteroids (Fig. 18). Whether these are energy-intensive or simple diffusive processes awaits further investigation.

5. *Transformation of Lotus japonicus to overexpress thiol synthetases*

5.1. Introduction

The techniques of plant transformation offer a powerful tool with which to perturb and elucidate interactions between metabolic processes. The availability of cDNAs encoding γECS, GSHS, and hGSHS from pea and bean nodules (Matamoros et al., 1999b; Moran et al., 2000) will allow us the upward or downward regulation of the enzymes involved in thiol synthesis. These studies will hopefully produce interesting information on the regulation of the pathway for GSH and hGSH synthesis, and on the role of thiols in N_2 fixation.

Like the model plant Arabidopsis, the legume *Lotus japonicus* possesses many biological and genetic advantages (Handberg and Stougaard, 1992; Jiang and Gresshoff, 1993). It has a small genome size (~ 350-450 Mb per haploid genome), is a true diploid with a low chromosome number (n=6), has a short generation time, large self-fertile flowers, large number of small seeds per pod, and, because of large flower size and apparent absence of incompatibility, is easy to cross sexually. Some lines like "Gifu" have the ability of high frequency regeneration from tissue cultures of root, hipocotyl, and leaf sections. Furthermore, the line is easily transformed by *Agrobacterium tumefaciens* and *A. rhizogenes,* facilitating insertional mutagenesis and gene tagging (Thykjaer et al., 1995; Stiller and Gresshoff, 1996). Nodules develop quickly (within 7 to 10 days) after inoculation with *Mesorhizobium loti*. The plant develops a good size, allowing biochemical analyses, necessary to transit from molecular biological studies to functional investigations.

Genetic and biological features make *L. japonicus* an attractive experimental organism. Initial studies by several laboratories studying N_2 fixation and nodulation have led to the proposal (Handberg and Stougaard, 1992) to use *L. japonicus* as a model for the study of determinate nodulation. Alternative model legumes, such as *Medicago truncatula* (Barker et al., 1990), exist for indeterminate nodulation.

5.2. Materials and methods

5.2.1. Binary vectors

In contrast to direct DNA uptake methods (Freeman et al., 1984; Fromm et al., 1985; Shillito et al., 1985) of plant transformation that require plant cell protoplasts, *Agrobacterium* is capable of infecting intact cells (An, 1985; Horsch et al., 1985). As a result, tissue culture limitations are much less of a problem in *Agrobacterium*-based transformation systems. Transformed plants can be regenerated more rapidly and more frequently when plant tissues are transformed directly without protoplast isolation. Another feature is that *Agrobacterium* is capable of transferring large fragments of DNA very efficiently without substantial rearrangement. Direct DNA uptake methods tend to induce rearrangements of the introduced DNA within transformed plants (Krens et al., 1985; Czernilofsky et al., 1986).

Agrobacterium introduces a segment of its tumor-inducing (Ti) plasmid, the T-DNA (transferred DNA), to the genome of a large number of gymnosperms and angiosperms. Transfer of the T-DNA from *Agrobacterium* to plants is dependent on the virulence (*vir*) functions outside the T-region of the Ti plasmid since, additional sequences outside the T-DNA are required for efficient T-DNA transfer, at least in some *Agrobacterium* strains.

The *Agrobacterium* binary vectors exploit the observation that the only *cis*-acting elements necessary for transfer are the left and the right borders of the T-DNA. The binary vector system consists of two plasmids, namely, the binary vector and the *vir* plasmid. The binary vector carries the region that will be transferred to plants (T-region), is small, easy to manipulate in *Escherichia coli*, and replicates both in *E. coli* and *Agrobacterium*. The large *vir* plasmid is an engineered Ti plasmid lacking the T-region but carries all the *vir* functions required for T-DNA transfer. When both plasmids are present in the same *Agrobacterium* cell, the genes products encoded by the *vir* plasmid will mobilize the transfer of the binary plasmid´s T-region to the plant cells.

Commonly the binary vectors consist of the following elements: **(1)** a origin of replication that allows the plasmid replication in both *E. coli* and *Agrobacterium*; **(2)** a gene that confers resistance to antibiotics both in *E. coli* and *Agrobacterium*; **(3)** a plant marker gene, located into the T-DNA, provides a selectable marker that functions in transformed plant cells; **(4)** the right and the left borders of the T-DNA; **(5)** the vectors also contain a polylinker with unique cloning sites between the T-DNA borders.

In our constructs we used the expression plasmid pZ-lbc3-pAnos (generously provided by Dr. Erik Østergaard, Institute for Molecular and Structural Biology, University of Aarhus, Denmark). It contains, in addition to the general elements of the binary vectors, the DNA carrying the soybean *lbc3* promoter with a double-enhancer sequence (fig. 19), which presumably confines gene expression to the nodule infected zone.

Figure 19. *The expression vector pZ-lbc3-pAnos*

5.2.2. Gene cloning and plant transformation

For transformation of *Lotus* to overexpress the bean nodule γECS (Matamoros et al., 1999b) and the pea nodule GSHS and hGSHS (Moran et al., 2000), the fragments (cloned into pCR II or PCR 2.1) containing the ORF for *γecs* (1.5 Kb; with and without the sequence coding the signal plastid peptide), the ORF for *gshs* (1.66 Kb) and the ORF for *hgshs* (1.49 Kb) were inserted into the plasmid pZ-lbc3-pAnos.

The binary vectors were introduced into *A. tumefaciens* strain 4404 by triparental mating. In this process the constructs (pZ-lbc3-pAnos plus fragment) were transferred from *E. coli* to *Agrobacterium* using the helper plasmid pKR2013.

In the following next months, the transformation of *Lotus* will be carried out as described by Stiller et al. (1997). The protocol is based on hypocotyl transformation by *A. tumefaciens,* and will allow us the effective production of large number of transgenic plants.

6. *Discusión general*

En este trabajo hemos estudiado detalladamente el metabolismo de tioles en nódulos de leguminosas. Se han seleccionado ocho especies de interés agronómico, cuatro con nódulos de tipo indeterminado (guisante, haba, alfalfa y lupino) y cuatro con nódulos de tipo determinado (soja, judía, "mungbean" y "cowpea"). El estudio comparativo entre especies con distintos tipos de nódulos resulta de gran interés, ya que los nódulos de tipo indeterminado y determinado difieren en características estructurales y metabólicas importantes (Hirsch, 1992).

En estudios previos (Escuredo y cols., 1996; Gogorcena y cols., 1997), la concentración de GSH+hGSH en los nódulos se estimó utilizando el ensayo enzimático descrito por Griffith (1980). Este método no distingue entre GSH y hGSH, y sobreestima la cantidad total de tioles presentes en los tejidos de las plantas productoras de hGSH, ya que la glutatión reductasa de levadura utilizada en el ensayo reacciona más rápidamente con hGSH que con GSH (Klapheck, 1988). Sin embargo, la técnica de HPLC con detector de fluorescencia nos ha permitido diferenciar ambos tioles en los diferentes tejidos y especies de leguminosas, cuantificar las actividades γECS, GSHS y hGSHS de forma precisa, y estudiar el metabolismo de los tioles en las plantas. Para esto último ha sido muy importante la determinación de tioles muy poco abundantes en los tejidos vegetales, especialmente Cys y γEC.

El contenido de tioles en los nódulos es considerablemente mayor que en las raíces y hojas (Tablas 4 y 5). Este hallazgo es importante ya que las hojas han sido consideradas tradicionalmente el principal lugar de síntesis de tioles en las plantas (Rennenberg, 1997). En general, los nódulos de tipo indeterminado contienen principalmente GSH, mientras que en los de tipo determinado el hGSH es el tiol predominante. Una excepción a este hecho es la presencia exclusiva de GSH en nódulos de "cowpea" (tipo determinado), lo cual sugiere que no existe una relación directa entre el tipo de nódulo y la presencia de GSH o hGSH. Asimismo, especies muy relacionadas filogenéticamente como "mungbean" (*Vigna radiata*) y cowpea (*Vigna unguiculata*) producen mayoritariamente hGSH o GSH, respectivamente. Similarmente, las hojas de alfalfa *(Medicago sativa*) contienen exclusivamente hGSH, mientras que las de la especie próxima *Medicago truncatula* contienen sólo GSH (Frendo y cols., 1999; Matamoros y cols., 1999b). Todas estas observaciones indican que, en contra de lo sugerido por otros autores (Klapheck, 1988), la distribución de tioles entre las diversas especies de leguminosas no tiene valor taxonómico.

Para determinar si los tioles eran importados de otras zonas de la planta o bien eran sintetizados genuinamente en los nódulos, se midieron las actividades tiol sintetasa implicadas en la biosíntesis de GSH y hGSH (Tabla 6). Los resultados demuestran que los nódulos sintetizan tioles y sugieren que éstos desempeñan un papel clave en la fijación de N_2. Esta hipótesis es apoyada por el hecho de que tanto en los nódulos de tipo determinado como en los de tipo indeterminado la concentración de GSH y hGSH es superior en la zona fijadora de N_2 (Figs. 8 y 9).

Otra cuestión importante fue dilucidar si en los nódulos existe un solo enzima, que sintetizaría indistintamente GSH y hGSH dependiendo de la disponibilidad relativa de Gly y βAla, o dos enzimas con diferentes afinidades por cada aminoácido. Nuestros resultados demuestran la existencia de dos enzimas, GSHS y hGSHS, cuya distribución, además, determina la abundancia relativa de GSH y hGSH en las diferentes especies y tejidos.

La existencia de dos enzimas que sintetizan específicamente GSH y hGSH se confirmó posteriormente a nivel molecular. En nódulos de guisante pudimos aislar dos cDNAs (*GSHS1* y *GSHS2*) con elevada homología con otras secuencias que codifican GSHS en plantas superiores (Moran y cols., 2000). El análisis RT-PCR reveló que *GSHS1* se expresa en nódulos y hojas, mientras que *GSHS2* sólo en nódulos. Ya que las hojas de guisante expresan exclusivamente GSHS (Tabla 10), *GSHS1* codifica una GSHS. En cambio, los nódulos de guisante expresan tanto GSHS como hGSHS (Tabla 6), por lo que *GSHS2* codifica una hGSHS. En judía se expresa en nódulos y hojas exclusivamente *GSHS2* , lo cual coincide con el hecho de que sólo se obtuvo un clon, que debe por tanto corresponder a una hGSHS (Tablas 6 y 10).

En guisante el análisis de las secuencias de cDNA y la utilización de programas de predicción pusieron de manifiesto la presencia en la proteína codificada por *GSHS1* de un péptido señal que presumiblemente dirige la proteína a las mitocondrias. Sin embargo, estos estudios no revelaron la existencia de ningún péptido señal en el enzima codificado por *GSHS2*, lo cual indica que es citosólico. En judía los resultados no son tan evidentes. Diferentes programas de predicción indican la posible existencia de un péptido señal que dirigiría el enzima codificado por *GSHS2* a los plastidios. Los resultados obtenidos en los estudios de fraccionamiento subcelular demuestran la existencia de al menos un isoenzima de la hGSHS en el citosol, aunque no hemos detectado actividad en plastidios y cloroplastos. Esto podría deberse a que la actividad hGSHS en los plastidios representa una pequeña proporción respecto de la total en los nódulos. Así, Klapheck y cols (1988) encontraron que únicamente un 17% de la actividad hGSHS en hojas de *Phaseolus coccineus* se localizaba en los cloroplastos. Otra explicación, no

necesariamente incompatible con la anterior, es que la isoforma plastidial es especialmente lábil y se inactiva durante la extracción. Los estudios de fraccionamiento subcelular también demostraron que la GSHS está localizada en el citosol y las mitocondrias (Fig. 16). Ésta es la primera vez que se detecta actividad GSHS en mitocondrias de plantas, lo cual no es sorprendente debido a que las mitocondrias son una fuente importante de radicales libres en las células vegetales (Dalton, 1995). El GSH podría actuar como antioxidante en estos orgánulos, ya que se ha descrito que la disminución del cociente GSH/GSSG está relacionada con un aumento del daño oxidativo al DNA mitocondrial (García de la Asunción y cols., 1996). Por otra parte, los estudios a nivel tisular demostraron que la hGSHS se localiza mayoritariamente en el córtex de los nódulos determinados, lo cual sugiere un papel específico del hGSH y de la hGSHS en los nódulos. Concretamente, es probable que exista un ciclo ascorbato-hGSH en los nódulos determinados (judía, soja, "mungbean"), y también en el córtex de algunos nódulos indeterminados (guisante), similar al ciclo ascorbato-GSH descrito en cloroplastos (Foyer y cols., 1994). Es además tentador pensar que dicho ciclo ascorbato-hGSH podría estar implicado en la eliminación de H_2O_2 generado a nivel de la barrera a la difusión de O_2 (Dalton y cols., 1998), localizada en el córtex interno (Minchin, 1997). De hecho, a este nivel se ha demostrado una gran abundancia de ascorbato peroxidasa (proteína y transcrito) y de actividad respiratoria (Dalton y cols., 1998; Matamoros y cols., 1999a).

El análisis de los cDNAs que codifican la γECS de nódulos de guisante y judía indica que este enzima es presumiblemente plastídico (Matamoros y cols., 1999b). Esta localización fue confirmada por los estudios de fraccionamiento subcelular (Fig. 16). Este hecho confiere a los plastidios no fotosintéticos un importante papel, hasta ahora ignorado, en la biosíntesis de tioles, así como en la defensa celular frente a las especies reactivas de oxígeno. En particular, hemos demostrado recientemente que, además de la γECS, los plastidios de los nódulos contienen ferritina (Matamoros y cols., 1999a) y FeSOD (Rubio y cols., 2000), dos importantes proteínas antioxidantes. Asimismo, Tang y Webb (1994) aislaron a partir de una genoteca de cDNA de nódulos de soja una secuencia que codifica una glutatión reductasa con un posible péptido señal que dirigiría el enzima a los plastidios.

Otro hallazgo novedoso en este trabajo fue que los bacteroides poseen elevadas actividades γECS y GSHS, así como un alto contenido de GSH, lo cual indica que los bacteroides son una fuente importante de GSH en los nódulos. Esto queda especialmente patente en los nódulos de las leguminosas productoras de hGSH. Por ejemplo, en la zona infectada de los nódulos de judía y soja la mayor

parte del GSH, si no todo, sería aportado por los bacteroides, mientras que el hGSH provendría de la fracción vegetal del nódulo.

También se analizó el efecto de la edad y el estrés sobre la concentración de tioles y las actividades tiol sintetasa de los nódulos. Se comprobó que tanto la senescencia natural como la inducida producen una disminución generalizada de tioles y de las actividades biosintéticas. Estos datos están en consonancia con trabajos anteriores, donde se describe un descenso general de las defensas antioxidantes y un aumento de los niveles de lípidos y proteínas oxidados (indicadores de estrés oxidativo) durante la senescencia nodular (Escuredo y cols., 1996; Gogorcena y cols., 1997; Evans y cols., 1999; Matamoros y cols., 1999a).

La obtención de plantas transgénicas con niveles alterados (sobreexpresión o inactivación) de los enzimas antioxidantes, incluidos los implicados en la biosíntesis de tioles (Rennenberg, 1997; May y cols., 1998a; Noctor y Foyer, 1998), han aportado datos importantes sobre su función y regulación (Foyer, 1994). La transformación de *Lotus*, una especie que por sus características ha sido propuesta como modelo para el estudio de la simbiosis rizobio-leguminosa (especialmente de las especies con nódulos determinados; Handberg y Stougaard, 1992), nos permitirá profundizar en el conocimiento de la regulación del metabolismo de los tioles en los nódulos.

7. *Conclusiones*

1. Los nódulos de leguminosas contienen concentraciones de tioles superiores a las de raíces y hojas de las mismas plantas. En particular, los tioles son especialmente abundantes en la zona fijadora de N_2. Ambas observaciones sugieren que el GSH y el hGSH desempeñan un papel importante en la fijación de N_2.

2. En general, el GSH predomina en los nódulos indeterminados y el hGSH en los determinados. Sin embargo, notables excepciones como *Vigna unguiculata* (especie con nódulos determinados que contienen exclusivamente GSH) sugieren que no existe una relación directa entre el tipo de nódulo y el tripéptido mayoritario. Asimismo, la abundancia relativa de GSH y hGSH no tiene valor taxonómico, ya que especies muy relacionadas filogenéticamente (*Vigna unguiculata* y *V. radiata*, o *Medicago sativa* y *M. truncatula*) muestran una composición muy diferente de los dos tripéptidos.

3. Los nódulos son el principal lugar de síntesis de tioles en las leguminosas. En los nódulos las rutas biosintéticas del GSH y hGSH comparten el primer enzima (γECS), mientras que el segundo enzima (GSHS o hGSHS) es específico para cada tripéptido. La presencia de GSH y hGSH en las diversas especies y tejidos de una misma planta viene determinada por la distribución de las correspondientes GSHS y hGSHS.

4. Los estudios de fraccionamiento subcelular de nódulos revelan que la γECS, el enzima regulador de la vía, se localiza principalmente en los plastidios, lo cual confiere a este orgánulo un papel clave en la síntesis de tioles y, en general, en la defensa antioxidante de los nódulos. La GSHS es predominantemente citosólica, aunque también puede encontrarse en las mitocondrias, mientras que la hGSHS se localiza mayoritariamente en el citosol y, probablemente, aunque en menor medida, en los plastidios. Asimismo, este último enzima es especialmente abundante en el córtex de los nódulos determinados, lo cual sugiere un papel específico del hGSH y la hGSHS en esta zona del nódulo.

5. La senescencia natural e inducida por estrés provoca un descenso general de la concentración de tioles y de las actividades tiol sintetasa. La disminución de tioles se debe también a un aumento en su degradación causado por el estrés oxidativo.

6. Los bacteroides constituyen una fuente importante de GSH en los nódulos. Este hecho es especialmente relevante en los nódulos de las especies productoras de hGSH, donde el GSH del nódulo (incluido córtex y zona infectada) es aportado exclusivamente por el bacteroide. Sin embargo, en estas últimas especies el hGSH también es captado por el bacteroide a través de la membrana simbiosomal.

7. Finalmente, en este trabajo se han aislado cDNAs con las secuencias codificantes completas de las tiol sintetasas de nódulos. Los análisis de las secuencias confirman la localización subcelular de los enzimas y revelan su elevada homología con las γECS y GSHS de otras plantas superiores. Con las secuencias de cada uno de los genes se están realizando construcciones con promotores específicos para transformar *Lotus japonicus*. Estos estudios nos permitirán llevar a cabo un análisis de la expresión y regulación de los genes y determinar el papel protector de los enzimas sobre la fijación de N_2.

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